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Chiral separations using bile salts in capillary electrophoresis

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CHIRAL SEPARATIONS USING BILE SALTS IN CAPILLARY ELECTROPHORESIS

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This dissertation is dedicated to my parents, Marge and Jim, my brother, John, and Elizabeth for their love, support and encouragement.

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ABSTRACT

CHIRAL SEPARATIONS USING BILE SALTS IN
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Micellar electrokinetic capillary chromatography (MECC) using surfactant solutions containing bile salts, polyoxyethylene ethers and methanol was used to produce chiral separations. A capillary electrophoresis instrument was assembled in the laboratory. Initial testing and optimization of the instrument involved the separation of organic acids using capillary zone electrophoresis techniques. Polymer coated capillaries were prepared and tested in the instrument. Bile salt solutions were characterized by viscosity and nuclear magnetic resonance. The effects of various solution conditions, such as pH, ionic strength and concentration of bile salt solutions on chiral resolution were investigated by MECC. Enhancement in chiral resolution was obtained using additives such as polyoxyethylene ethers and methanol in bile salt micellar solutions. Fluorescence spectroscopy using pyrene as a probe was utilized to determine the relative hydrophobicities of the aggregates formed in solutions containing mixtures of bile salt and polyoxyethylene ether. Correlations between micellar hydrophobicity for these solutions and chiral resolution for bi-2-naphthol, verapamil, norverapamil, gallopamil and BAYK8644 are presented.

CHAPTER 1

INTRODUCTION

1.1 Introduction

The first reference to practical applications of capillary electrophoresis is considered to be a paper published in 1981 by James Jorgenson and Krynn Lukacs entitled "Zone Electrophoresis in Open-Tubular Glass Capillaries"(1). This is a field which has experienced tremendous growth and innovation in the past decade. This field will continue to expand for many years to come, due to the great variability in the possible separation modes and wide applicability to separation problems. An STN Chemical Abstracts on-line search conducted in August 1994 yielded over 500 publications in the field of capillary electrophoresis. It is estimated that over one thousand laboratories worldwide are using some form of capillary electrophoresis (2-5). The popularity and expansion of this field of separations is largely due to the high efficiency obtainable with such intrinsic simplicity. At a time when separation methods are becoming more complex and resource intensive, capillary electrophoresis offers a method which is both simple and relatively inexpensive to use. Applications of capillary electrophoresis range from separations of small molecules and ions to complex mixtures of proteins. Chiral separations have been a very active area of research in capillary electrophoresis. In the field of pharmaceutical research, where it is absolutely necessary to separate and characterize each individual enantiomer of chiral drugs, capillary electrophoresis has become a very useful analytical separation method. This chapter discusses the theoretical considerations and fundamentals of capillary zone electrophoresis, the different types of techniques which make up the field of capillary electrophoresis, and the applications of several

of these techniques to chiral separations.

1.2 Basics of Capillary Electrophoresis

1.2.1 Theory

Electrophoresis is a process for separating charged molecules based on their movement through a fluid under the influence of an applied electric field. When a potential is applied between the two ends of a sufficiently small diameter capillary tube filled with a buffer solution, a unique "pumping action" occurs that is termed "electroosmotic flow". This flow is the basis for its high efficiency separations. Positive ions from the buffer solution interact and effectively stack up near the negatively charged inner surface of the glass capillary, forming an electrical double layer (6). When the potential is applied, the positive ions are attracted towards the negative electrode or cathode. Positive ions outside of the rigid inner Helmholtz layer undergo electrophoresis in that direction. The concentration of positive ions is greatest at the surface which surrounds the buffer solution in the capillary tube. This allows these ions to pull the entire solution through the capillary tube in a sheath flow profile. This sheath flow possesses a near flat flow profile which does not contribute significantly to band broadening. Thus, the action of electroosmotic flow provides the simplicity and efficiency advantage over other separation techniques. No pumping devices are necessary to move the solution through the tube and the flow profile is nearly ideal. Detection is normally conducted on-column to eliminate zone broadening caused by joints, fittings and connectors.

To make a qualitative assessment of the separation ability of capillary electrophoresis the principles of separation theory must be considered. Giddings defines separation as the science of maximizing separative transport relative to dispersive transport (5,7). One of the

main advantages of capillary zone electrophoresis is that there is no need for a pressure-driven flow which usually results in a parabolic flow profile and thus band broadening. Since open-tubular capillaries of small internal diameter are employed, band broadening due to resistance to mass transfer and heating effects are minimized. Consequently, the only factor significantly contributing to band broadening during the separation process is longitudinal diffusion (1, 8-10). The migration velocity, v, in electrophoresis is given by:

$$v = \mu E = \frac{\mu V}{L} \tag{1.1}$$

and the mobility, μ, is given by:

$$\mu = \mu_{ep} + \mu_{eo} \tag{1.2}$$

where E is the field strength, V is the voltage applied across the capillary, L is the capillary length, μ_{ep} is the electrophoretic mobility, and μ_{eo} is the electroosmotic mobility. The time required for a solute to migrate from one end of the capillary to the other is the migration time, t, and is given by:

$$t = \frac{L}{v} = \frac{L^2}{\mu V} \tag{1.3}$$

Assuming that the only contribution to band broadening is longitudinal diffusion, the variance of the migrating zone width, σ^2, can be written as:

$$\sigma^2 = 2Dt = \frac{2DL^2}{\mu V} \tag{1.4}$$

or

$$\sigma = \left[\frac{2DL}{\mu E}\right]^{1/2} \tag{1.5}$$

where D is the diffusion coefficient of the solute (1,8-16). The number of theoretical plates, N, is given by:

$$N = \frac{L^2}{\sigma^2} = \frac{\mu V}{2D} \tag{1.6}$$

The efficiency is therefore based on applied voltage, not capillary length. Maximum efficiency and short analysis times should be obtained with the use of high voltages and short columns, provided there is efficient heat dissipation.

When capillary electrophoresis is conducted in a column constructed from glass or other materials which provide a charged surface, the effects of electroosmotic flow on migration velocity (v), migration time (t), zone variance (σ^2), and theoretical plates (N) must be considered. The potential across the layers generated at the silica-solution interface is called the zeta potential, denoted by ζ, and is given by:

$$\zeta = \frac{4\pi\eta\mu_{eo}}{\varepsilon} \tag{1.7}$$

where η is the viscosity, ε is the dielectric constant of the solution, and μ_{eo} is the coefficient for electroosmotic flow (17,18). Electroosmotic flow affects the amount of time a solute would take to migrate through the capillary, and therefore, may indirectly affect both efficiency and resolution.

According to the equation for the migration velocity, all ions will migrate in the same

direction if the coefficient for electroosmotic flow is greater in magnitude and opposite in direction to the electrophoretic mobility of all anions in the buffer. Non-ionic species will be carried by the electroosmotic flow and migrate to one end of the capillary. Under these conditions, the observed migration order would be cations, neutrals, and anions, with cations having the shortest migration times. Since the separation is based on differential electrophoretic migration, neutral species are not separated in CZE.

The resolution of two zones in electrophoresis is given by the equation:

$$R = \frac{1}{4(2)^{1/2}} (\mu_{ep,1} - \mu_{ep,2}) \left[\frac{V}{LD(\mu_{ep} + \mu_{eo})} \right]^{1/2} \quad (1.8)$$

where $\mu_{ep,1}$ and $\mu_{ep,2}$ are the electrophoretic mobilities for the two solutes and μ_{ep} is the average of $\mu_{ep,1}$ and $\mu_{ep,2}$ (1, 8-12). According to the resolution equation, the highest value for resolution is obtained when $\mu_{eo} = -\mu_{ep}$. In this case the analysis time approaches infinity. Thus a balance must be found in which the solution and instrumental conditions allow for the best separation of a group of analytes with a given set of electrophoretic mobilities in a reasonable period of time.

1.2.2 Capillary Zone Electrophoresis

The first and most basic form of capillary electrophoresis (CE) is capillary zone electrophoresis (CZE). The instrument used for this technique includes a high voltage source, a capillary tube, a uv-vis variable wavelength detector, signal recording and processing equipment, and a device to control the voltage and monitor the current. The ends of the capillary are immersed in vials containing buffer in which the electrodes from the high voltage source are placed. Using a capillary which has a negative surface charge, such as fused silica,

electroosmotic flow is observed in the direction from the anode to the cathode. Injections are made at the anode end in this technique. On-column detection is performed near the negative electrode or cathode end. Chapter 2 details a series of improvements which were made to a laboratory-assembled CE instrument. The results of several studies conducted to optimize the solution conditions for the separation of a group of organic acids by CZE are presented in that chapter.

Injection. Injections can be performed in several ways. These include electrokinetic and several variations of hydrodynamic methods. In the electrokinetic method, the anode and injection end of the capillary are placed in the solute solution, while the cathode and detection end of the capillary are placed in an appropriate buffer solution. Injection is accomplished by applying the voltage for 1 to 30 seconds. The voltage applied for injection (5-10 kV) is generally less than the applied voltage during the electrophoretic run (10-30 kV). Hydrodynamic injection can be accomplished in four ways: 1) by elevating the capillary at the sample end, permitting sample introduction by siphoning; 2) by applying pressure on the liquid in the sample vial; 3) by applying vacuum on the detector end of the capillary; or 4) by using a split flow syringe to reduce the volume introduced into the capillary (4,5). For a hydrodynamic syphoning type injection, with the use of a 50 micron ID capillary, the amount injected is approximately 0.17 nL/s at a height of 10 cm (5). Injected volumes for other methods are generally within an order of magnitude of the volume injected using the hydrodynamic method, allowing for nanoliter quantities of sample to be injected on-column.

Detection. Detection is accomplished on-column when using fused silica capillaries by using a portion of the capillary as a flow cell. The polyimide coating in a portion near the

anode end of the capillary is removed to provide a uv transparent flow cell. The light exiting the monochromator is directed on this portion of the capillary and the light passing through the capillary is directed on to the detector. The major limitation of this type of detection scheme is the small spectroscopic path length provided by the capillary flow cell. The technique requires the small ID capillary tubing for the electroosmotic flow to be generated. The challenge is obtaining sensitive detection using this small path length. Solutes must have significant molar absorbtivity at the wavelength chosen for detection. The most commonly used wavelength range for detection has been 200 to 210 nm. Detection is performed in this range due to the fact that most of the compounds studied have a significant absorbance in this range compared to the observed background and noise.

1.3 Variations of Capillary Electrophoresis

Capillary electrophoresis (CE) is the name given to a family of separation techniques based on an electrophoretic, or charge based separation mechanism, conducted in a capillary tube. Several distinct capillary electroseparation methods have developed from the principles of Capillary Zone Electrophoresis (CZE). These methods include:

1.3.1 Capillary Gel Electrophoresis (CGE)

The CGE separation mechanism is based on differences in solute size as analytes migrate through pores of the gel-filled column. The pore size is determined by the concentration of polymeric agent or polymer network and the three dimensional structure of the matrix. The porous structure of the gel provides effective separations of macromolecules based on molecular size. Recent applications of CGE have included the separations of antisense oligonucleotides with polyacrylamide gel filled capillaries using uv detection (19)

and antisense DNA analogues with specially pretreated polyacrylamide gel filled capillaries using laser-induced fluorescence detection (20).

1.3.2 Micellar Electrokinetic Capillary Chromatography (MECC or MEKC)

The MECC separation mechanism is based on solutes partitioning between the micellar pseudophase and the solution phase. Larger differences in migration times of the two phases increases the resolving power. The technique provides a method to resolve mixtures of neutral molecules as well as enhanced separations of charged molecules by CE. This method has also found widespread applications in chiral separations of neutral and charged enantiomeric pairs using chiral surfactants or pseudophases containing chiral agents. Due to the ability of the technique to separate neutral and charged molecules, this has been one of the most active areas of research and applications in CE (2-5). Recent applications of MECC to pharmaceutical separations have included the determination of drugs in biofluids using micellar sodium dodecyl sulfate (SDS) solutions (21) and the separation of eight cardiovascular drugs using SDS solutions with organic modifiers (22).

1.3.3 Capillary Electrochromatography (CEC)

The capillary for CEC is packed with a chromatographic packing material which can retain solutes by the normal distribution equilibria on which liquid chromatography depends. The electroosmotic flow generated by the applied voltage is used in place of traditional pumping systems. The technique was used to demonstrate increased resolution over traditional liquid chromatographic methods. This was reported for a series of naphthalene sulfonic acids (23) and the drug Isradipin and by-products (24). The increased variability and difficulty due to micro-manipulation of column packings have prevented widespread

applications.

1.3.4 Capillary Isoelectric Focusing (CIEF)

In isoelectric focusing, compounds such as proteins are separated based on their isoelectric points or pI values. The protein samples and a solution that forms a pH gradient are placed inside the capillary when isoelectric focusing is performed using a CE instrument. The anodic end of the column is placed into an acidic solution, and the cathodic end in a basic solution. Under the influence of the electric field, the charged proteins migrate through the medium until they reside in a pH region where they become electrically neutral and therefore stop migrating. Consequently, the zones are "focused" until a steady state is reached. After focusing, the separated zones can be forced to migrate through the column using pressurized flow. Solutes are detected as they pass by the detection window under the pressurized flow. Recent advances in the field have concentrated on modifying the surface chemistry of coated and uncoated fused silica capillaries to decrease nonspecific binding of peptides, proteins, and antibodies to the capillary wall (25). The research presented in Chapter 3 details the investigations conducted to create and analyze polymer coated capillaries for CE.

1.3.5 Capillary Isotachophoresis (CITP)

Isotachophoresis is a widely used separation technique which separates analytes on the basis of their electrophoretic mobilities. Commercial instruments designed specifically for isotachophoresis are available, yet CITP separations can be performed on most CE systems. The main feature of CITP is that it is performed in a discontinuous buffer system. The sample migrates between two solutions of different ionic mobility, the leading and terminating electrolytes. Sample components condense between leading and terminating constituents,

producing a steady state migration profile composed of consecutive sample zones. Like CZE, CITP can be used for the separation of both cations and anions. Unlike CZE, separate CITP runs are required for the determination of both cations and anions. Recent applications of this technique have involved using a CITP methods for preconcentration of complex protein mixtures for CZE separations (26-29).

1.4 Chiral Separations Using Capillary Electrophoresis

Capillary electrophoresis has some distinct advantages over other separation methods for chiral separations. CE techniques generally have ultra-high separation efficiencies, easy exchanges of separation media, and extremely small volumes of the sample and the media. This means that, in the development of a CE chiral separation method, one can easily alter the separation solution to find the optimum separation medium and can also use an expensive chiral selector in small quantities. Several reviews of this field (30-38) have described the wide range of chiral applications which have been demonstrated. Applications in chiral CE are dominated by separations using various cyclodextrin compounds as a chiral selectors. These applications include using neutral, charged and derivatized cyclodextrins as buffer additives for CZE and MECC, and immobilized cyclodextrins in CGE and CEC. Other chiral selectors include crown ethers, proteins, polysaccharides and chiral micelles (30-38).

One of the most interesting chiral micellar systems used in CE are the bile salts (39-45). Chiral separations have been reported using bile salts as a pseudophase in MECC for only a few compounds, including: carboline derivatives(39), dansylated amino acids (40), binaphthyl derivatives (39, 43, 44), trimetoquinol and analogues (39, 41, 42), and diltiazem and analogues (39,41). Bile salt micellar solutions have several properties which make them

an ideal micellar pseudophase for MECC when using the most common type of CE system: bare fused silica capillaries and uv detection. These include: low uv absorbance, low critical micellar concentration (CMC), and micelles that possess a net anionic charge. The aggregate properties of bile salt solutions has been an active area of research for several decades due to the physiological importance of bile salts. A great deal is known about the behavior of bile salts in aqueous solutions (46).

The research presented in Chapters 4, 5 and 6 details the results of investigations conducted using knowledge of the aggregate properties of bile salt solutions to provide enhanced chiral separations and expand the applications of bile salt MECC. Results obtained from studies of chiral MECC separations of dl-laudanosine and ±bi-2-naphthol using bile salt solutions are presented in Chapter 4. Results of investigations of the factors affecting aggregation of bile salt solutions using viscosity and NMR techniques are also presented in Chapter 4 and correlated to the observed chiral separations. The results of investigations into using three different polyoxyethylene ethers and methanol as additives in bile salt solutions to enhance the observed chiral resolution of verapamil and related compounds are presented in Chapter 5. Fluorescence spectroscopic studies of solutions containing mixtures of bile salts and polyoxyethylene ethers using pyrene as a probe molecule are presented in Chapter 6. Further investigations of the chiral separations of verapamil and other compounds provided the correlations between fluorescence spectroscopic data and chiral resolution presented in Chapter 6.

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CHAPTER 2

CONSTRUCTION AND TESTING OF A CE INSTRUMENT

2.1 Introduction

A capillary electrophoresis (CE) instrument can be assembled in the laboratory from a conventional HPLC uv-vis absorbance detector and less than one thousand dollars in additional equipment and supplies. This chapter details the requirements of the components of a CZE instrument, presents the steps taken to optimize a laboratory assembled CE unit, and then presents the results of several studies of the separations of mixtures of phenolic acids. The requirements and limitations of this instrument are based on the use of a capillary tube as the column. A detector designed for use in high performance liquid chromatography can be modified for use in a CE instrument. A high voltage source (up to 20 or 30 kV) with low current output (1 mA) is available from many manufacturers. The voltage can be regulated by a controller purchased with the source, a controller constructed separately, or with a computer having appropriate software and hardware. The output from the detector must be recorded and processed. The remaining components include electrodes, switches, cables and a containment box.

The CE instrument was assembled and initial tests performed. A set of test analytes was chosen to learn more about the experimental parameters of CZE. The literature in CZE was not specific with regards to many experimental details of this technique at the beginning of these investigations. It was necessary to show that the laboratory assembled CE instrument could be used to successfully investigate the important parameters of the technique. The test solutes chosen were several phenolic acids which had been separated

using high performance liquid chromatography (1). The applied voltage in CZE is much like the flow rate in HPLC. The effect of the rate of the migration in the free solution capillary electrophoresis on the separation profiles of the phenolic acids was investigated.

The pKa's of the phenolic acids studied are in the range of 4.2 to 4.5. Most experiments in the literature used buffers near pH 7. It was proposed that as the buffer pH was lowered from 7, the fraction of the acid which was in the charged, or conjugate base form, would be lowered and migration times would drop. It was also reported in the literature that the electroosmotic flow was reduced as the pH was lowered (2).

2.2 Requirements

Capillaries. The most common type of capillary tubing used in CZE is fused silica. The capillary has an internal diameter of 50 to 150 microns, with an external polyimide coating to provide mechanical strength, yet allow flexibility. The minimum length of the capillary is limited by the instrumental setup and is generally about 20 cm. The maximum length is dictated by the need for reasonable migration times and is generally about 100 cm. Fused silica is uv transparent when the polyimide coating is stripped away to provide a detection window. Detection can be performed on-column by monitoring the absorbance through this section of the capillary tube.

Detection. Uv-vis detectors are the most common in CE, although the use of electrochemical (3,4), fluorescence (5,6), mass spectrometric (7) and nuclear magnetic resonance (8) detectors has also been reported. A uv-vis detector designed for use in high performance liquid chromatography (HPLC) can normally be modified for use with a CE system. Appropriate adjustments in the slit width must be made to record the narrow solute

bands characteristic of many CE separations. A capillary holder must be fashioned which allows alignment of the "flow cell" portion with the light aperture and the light path of the detector. Care must be taken since the portion of the capillary which will act as the flow cell, where the polymer coating has been removed, is brittle and breaks easily with bending. The capillary holder must be sufficient to hold the capillary firmly on either side of the window to prevent breakage when the portion of the capillary outside of the detector is moved for assembly or during injections. The capillary holder must also hold the capillary tightly enough to prevent external vibrations from causing the flow cell portion to vibrate during operation and disturb detection.

Power Supply and Regulation. The output from the high voltage source must be constant and accurately controlled. A voltage of 30 kV is generally found to be the upper limit for which an Ohm's law plot adheres to reasonable linearity for these small diameter capillaries. Several manufacturers have high voltage sources available which deliver voltages in the 0 to 30 kV range with good precision. A system must be constructed which allows the voltage and current of the output of the power supply to be monitored. Improved systems allow for computer control of the voltage, voltage ramps and precisely timed electrokinetic injections. The voltage is delivered to the vials of solution through an inert and highly conductive material such as platinum metal electrodes.

System Requirements. For operator safety, the apparatus should be contained within a box with an interlock installed, such that opening the box shuts off the high voltage. A switch is required to turn on and off the high voltage from outside the box. It was determined from our setup the cooling of the capillary and electronics inside the box is of the utmost

importance. Cooling can be accomplished by forcing air into the box. Recording of the signals from the detector is most conveniently accomplished externally from the box using a strip chart recorder, integrator, computerized data acquisition system, or a combination thereof.

2.3 Validation of a CE Instrument

Detection. The detection in CE is limited by the narrow pathlength of the capillary. The detection limit of the system can be established using a chromophore with known absorbance properties. The column can be filled with a standard solution of the chromophore in an appropriate buffer for CE and the absorbance measured at the detector. Successive dilutions of the standard solution in the buffer will allow the determination of the detection limit.

Aperture Selection. The aperture determines the length of the flow cell in which absorbance measurements are made. This distance should be sufficiently small to accurately record the individual solute bands. This was accomplished by making the length of column through which light is projected, or the slit width in the capillary holder, smaller than the width of an electrophoretic band on the column. The theoretical length of a solute band after injection is dependent upon the injection method. These lengths were calculated for a hydrodynamic and an electrokinetic injection and the aperture length was adjusted to be less than the width of the solute bands. Typical bands were approximately 400μm. The aperture lengths used were between 100μm or 300μm.

Electrokinetic vs. Hydrodynamic Injections. Hydrodynamic injections of the analyte containing solutions are conducted by placing the capillary end in the analyte solution and

raising the solution to a measured height for a specified time. A syphoning action draws a specific amount of analyte solution into the column. Typical heights and injection times are 5 to 20 cm and 5 to 30 sec, respectively. If a hydrodynamic injection is conducted at a height of 10 cm using a 50 μm ID column, 0.17 nL/s is introduced into the column (9). A 5 s injection would produce a solute band on the column with a length of 433 μm.

Electrokinetic injections are conducted by inserting the capillary end and electrode into the analyte solution and applying a voltage for a specified time. Both electrophoresis and electroosmotic flow affect the introduction of analytes into the column. The voltages used for injection are generally less than the voltage used for separations. Typical injection voltages and times are 5 to 10 kV and 5 to 20 sec, respectively. The length, l, of a sample zone injected using an electrokinetic injection is given by:

$$l = (\mu_{eo} + \mu_{ep}) \frac{V_i t_i}{L} \qquad (2.1)$$

where μ_{eo} is the electroosmotic mobility of the sample solution, μ_{ep} is the electrophoretic mobility of the sample molecule, V_i is the injection voltage, t_i is the injection time, and L is the length of the capillary (10).

Voltage Selection. The optimum voltage for a given separation depends on many factors. The upper voltage limit for a given buffer solution can be determined by observing the current for a range of voltages. An Ohm's law plot is a plot of observed current versus voltage. The highest recommended voltage where no negative effects due to joule heating is determined when a voltage is reached where a positive deviation in current is observable.

Voltage Ramps. Creating a gradient profile in the applied voltage versus time may

provide some of the same advantages for CE that have been observed in HPLC. The ability to ramp the voltage could also provide a convenient method for the analysis of complex mixtures with larger differences in electrophoretic mobilities.

2.4 Experimental

2.4.1 Instrumentation

The detector used for these studies was a variable wavelength uv-vis absorbance detector model AD-200 from SpectroVision, Inc. (Chelmsford, MA). The capillary was 50 μm ID fused silica from Polymicro Technologies (Phoenix, AZ). The high voltage power source was a 30 kV power supply model MJ30P400, from Glassman High Voltage, Inc. (Whitehouse Station, NJ). The output of the detector was recorded on a strip chart recorder, model BD40 from Kipp and Zonen (Holland), and a model 3390A integrator from Hewlett Packard (Wilmington, DE). A voltage control device was constructed by Mr. Richard Sweet of the University Instrumentation Center which allowed the applied voltage to be adjusted. The applied voltage and observed current could be monitored through separate outputs of this device. The detector and power supply were contained in a plexiglass box. A diagram of the instrumental setup is given in Figure 1-1. In the initial studies a 400 μm slit which was supplied with the detector was installed. For the final studies using smaller injections, a 100 μm pinhole was used as a slit.

For the voltage ramp investigations an analog to digital conversion board, model 208, from IBM Instruments (Danbury, CT) and an AT&T model 6300 Personal Computer were used. The program used to control the voltage, CZE.PAS, was written in PASCAL and developed by Dr. Barry Wythoff. The analog to digital conversion board was connected to

the voltage control device.

Instrumental Improvements. The instrument was contained in a plexiglas box having dimensions of 45 cm high, 77 cm wide and 45 cm deep. A removable side, 45 cm by 77 cm, was attached with hinges which allowed it to be opened and folded onto the top. A plexiglas box was constructed within the box to contain the high voltage power supply and provide a shelf at a height of 20 cm for the detector. An arm was constructed which held the capillary and anode with a rubber stopper. The arm was capable of rotating to lift the capillary and electrode simultaneously to a height of 10 cm. A compact high power fan was installed in the box to provide ventilation. A second fan was placed outside the box and aimed across the capillary and detector.

Alignment and Optimization of the Detection Window. A capillary holder was provided with the SpectroVision detector and underwent many modifications to improve physical stability of the capillary, alignment of the light path and reductions in the aperture width. A diagram of the capillary holder and the light path are given in Figure 1-2. Alignment of the capillary was conducted by: 1) removing the capillary holder from the detector, 2) removing the capillary alignment plate, 3) placing the capillary holder containing the capillary under a microscope with the bottom light of the microscope following through the light path in the capillary holder, 4) moving the position of the aperture to focus the light path on the center of the capillary tube, 5) securing the aperture and capillary in place with electrical tape, 6) replacing the capillary alignment plate, 7) verifying the alignment of the light path using the microscope, and 8) remounting the capillary holder in the detector. Steps 2 through 7 were often repeated several times to optimize the alignment due to movement of the capillary while

securing the capillary alignment plate.

2.4.2 Chemicals

Reagent grade sodium phosphate ($Na_3PO_4 \cdot 12H_2O$), potassium phosphate ($K_2HPO_4 \cdot 12H_2O$), phosphoric acid (85%), and sodium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ). 4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid) and 3,4-dihydroxybenzoic acid (protocatechuic acid) were obtained from Sigma Chemical Co. (St. Louis, MO). 4-Hydroxybenzoic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI). 4-Hydroxy-3-methoxybenzoic acid (vanillic acid) was obtained from Nutritional Biochemicals Corp. (Cleaveland, OH). 1,3,7-Trimethylxanthine (caffeine) was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ).

2.4.3 Methods

Injections. Electrokinetic injections were performed at 10 kV for 5 to 10 s. Hydrodynamic injections were performed at a height of 10 cm for 5 to 10 s.

Buffers. Phosphate buffers were prepared from solid disodium phosphate and potassium phosphate. Buffers were prepared by dilutions of these two solutions to create a buffer at the desired pH and an ionic strength of 0.01. Higher ionic strength buffer solutions were prepared by the adding of the appropriate amount of solid sodium chloride.

Samples. Sample solutions were prepared at analyte concentrations of 1.0 mg/mL in solutions of the same buffers which the electrophoresis experiments were conducted.

2.4.4. Separations of Organic Acids.

Figure 2-3 is a plot of observed retention times versus applied voltage for syringic acid, protocathuic acid, vanillic acid, 4-hydroxybenzoic acid and caffeine. The voltages

investigated in this study were: 10, 15, 20 and 25 kV. The phosphate buffer solutions for the capillary electrophoresis experiments and samples were prepared at a pH of 6.0 and an ionic strength of 0.01.

Figure 2-4 is a plot of observed retention times versus buffer pH for syringic acid, protocathuic acid, vanillic acid, 4-hydroxybenzoic acid and caffeine. The solutions investigated were at a pH of 5.0, 6.0 and 7.0. The phosphate buffer solutions for the electrophoresis and samples had an ionic strength of 0.01. All electrophoretic data for this study were acquired at 10 kV. Two electropherograms were acquired under the same conditions as this study at a pH of 7.0 after using different injection techniques. An electropherogram acquired after a 5 s hydrodynamic injection at a height of 10 cm is given in Figure 2-5. An electropherogram acquired after a 5 s electrokinetic injection at 10 kV is given in Figure 2-6.

2.5. Results and Discussion

2.5.1. Instrumental Improvements

Modifications were made to the instrument to improve the reproducibility and detection limit of the system. The instrument was moved into an air conditioned environment. An additional fan was placed outside the box with the airflow directed over the column and the top of the detector. These additional steps towards thermal stability and instrument cooling greatly improved the stability of the baseline absorbance.

The detector was equipped with a capillary column holder for CE applications in both sample and reference cells. Jumps in the baseline of 0.005 absorbance units were occurring at random time intervals. These seemed to correlate to mechanical shock to the instrument

and benchtop vibrations. The capillary column holder was modified to hold the capillary with greater mechanical stability. Improvements in the capillary entrance and exit ports in the detector were also made to increase capillary stability.

Improved baseline signal to noise and detection limit were also accomplished through optimization of detector settings for CE applications. With the small peak widths observed in CE the response time was set on the lowest setting. Detection was more sensitive when the light intensity observed by the reference photodiode was greater than that observed by the sample photodiode at 210nm. A 300 by 50 nm slit was used in the capillary holder on the reference side without a capillary installed.

The improvements to the plexiglas box helped to improve reproducibility. The shelves provided easier access to the anode and cathode. The arm was capable of rotating to lift the capillary and electrode simultaneously to a height of 10 cm for changing buffer and sample vials and performing precise hydrodynamic injections. The enclosure of the high voltage source provided an assurance of safety while working with aqueous buffers inside the box.

Voltage Ramps. The voltage ramp program was evaluated and found to operate effectively. Electrokinetic injections could be precisely timed. The voltage could be held constant or incrementally increased over a user-defined time interval. Application of the voltage ramp program was not necessary for the baseline separation of the organic acids.

2.5.2. Separations of Organic Acids.

The effect of applied voltage on the retention times of four organic acids and caffeine can be interpreted from the plot given in Figure 2-3. At a pH of 6.0 the acids are above their pKa values and are negatively charge. Thus, as anions, the compounds would be affected by

electrophoresis in the direction of the anode and electroosmosis in the direction of the cathode. The caffeine is expected to be neutral and migrate with the electroosmotic flow. The compounds migrate as predicted and the best resolution is observed at an applied voltage of 10 kV. The dependence of migration times on the inverse of the applied voltage is shown in Figure 2-3.

The effect of buffer pH on the retention times of the four organic acids and caffeine can be interpreted from the plot given in Figure 2-4. Retention times of the acids are lower using a buffer at a pH of 5.0. As the pH of the buffer approaches the pK_a values of the acids, the acids become less anionic and less affected by the electrophoresis, and the retention times decrease. Excellent resolution is observed using buffers with pH values of 6.0 and 7.0.

2.5.3. Injection Technique.

A comparison of hydrodynamic and electrokinetic injection methods for the organic acids can be made by comparing the electropherograms given in Figures 2-5 and 2-6, respectively. The electrokinetic injection is shown to inject a longer, less focused, band onto the column than the hydrodynamic injection. Theoretically, the length of a neutral solute band injected on the column for the 5 s hydrodynamic injection at a height of 10cm is 433μm, and for the 5 s electrokinetic injection at 10 kV is 416μm. Thus, there is clearly more band broadening associated with injection for the electrokinetic method than the hydrodynamic method for anionic compounds.

2.6. Conclusions.

The modifications to the capillary electrophoresis instrument improved the performance of the system. Reproducible hydrodynamic and electrokinetic injections were

accomplished using this instrumental setup. Temperature control of the detector provided a steady baseline absorbance signal. Baseline separation of four closely related hydroxybenzoic acids was accomplished with the improved system. The effects of voltage and pH were successfully investigated.

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Figure 2-1. Schematic Diagram of Laboratory Assembled Instrument

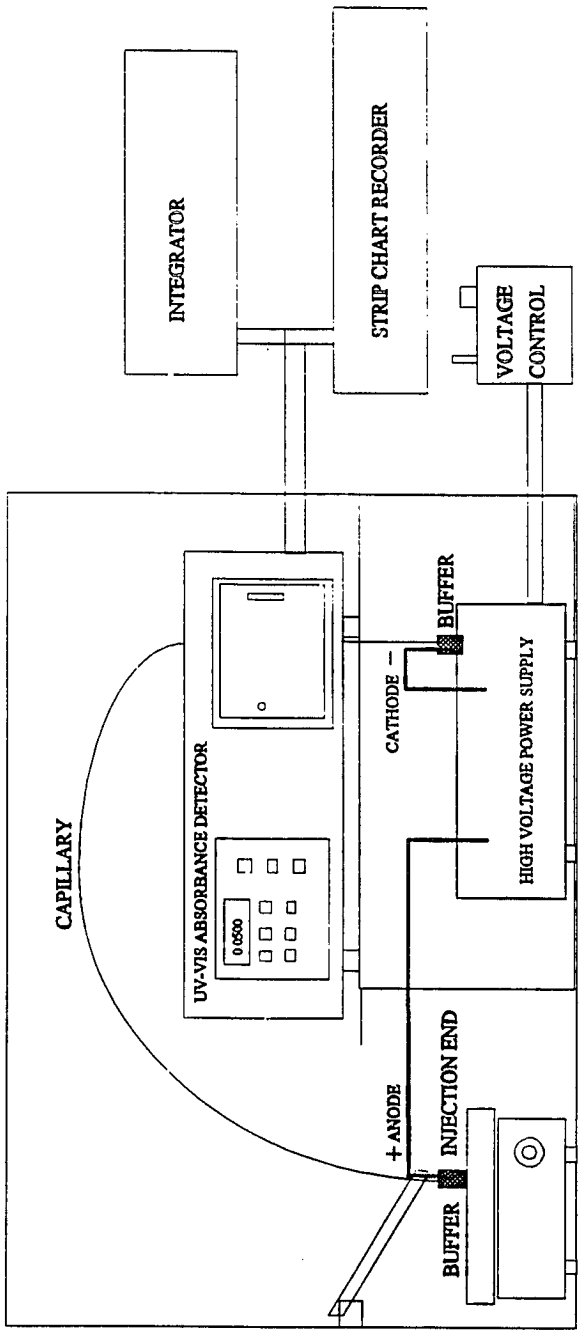
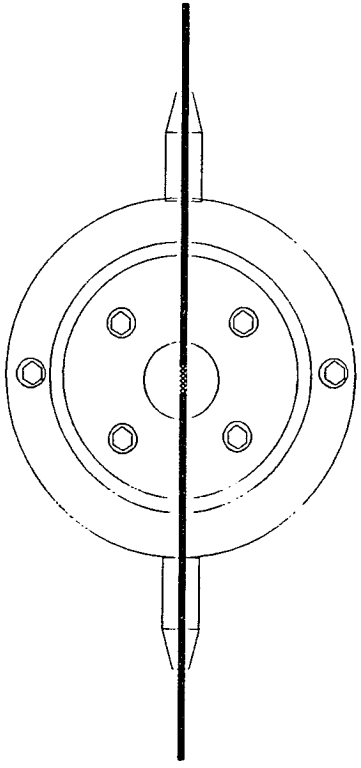
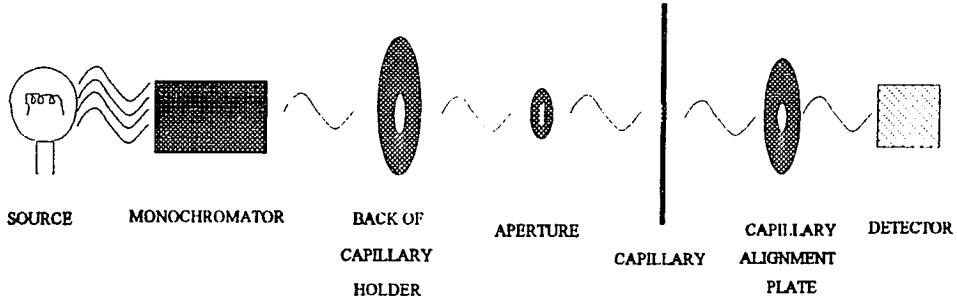


Figure 2-2. Capillary Holder and Light Path



CAPILLARY HOLDER



LIGHT PATH THROUGH CAPILLARY HOLDER

Figure 2-3. Effect of Voltage on Retention Times of Organic Acids and Caffeine

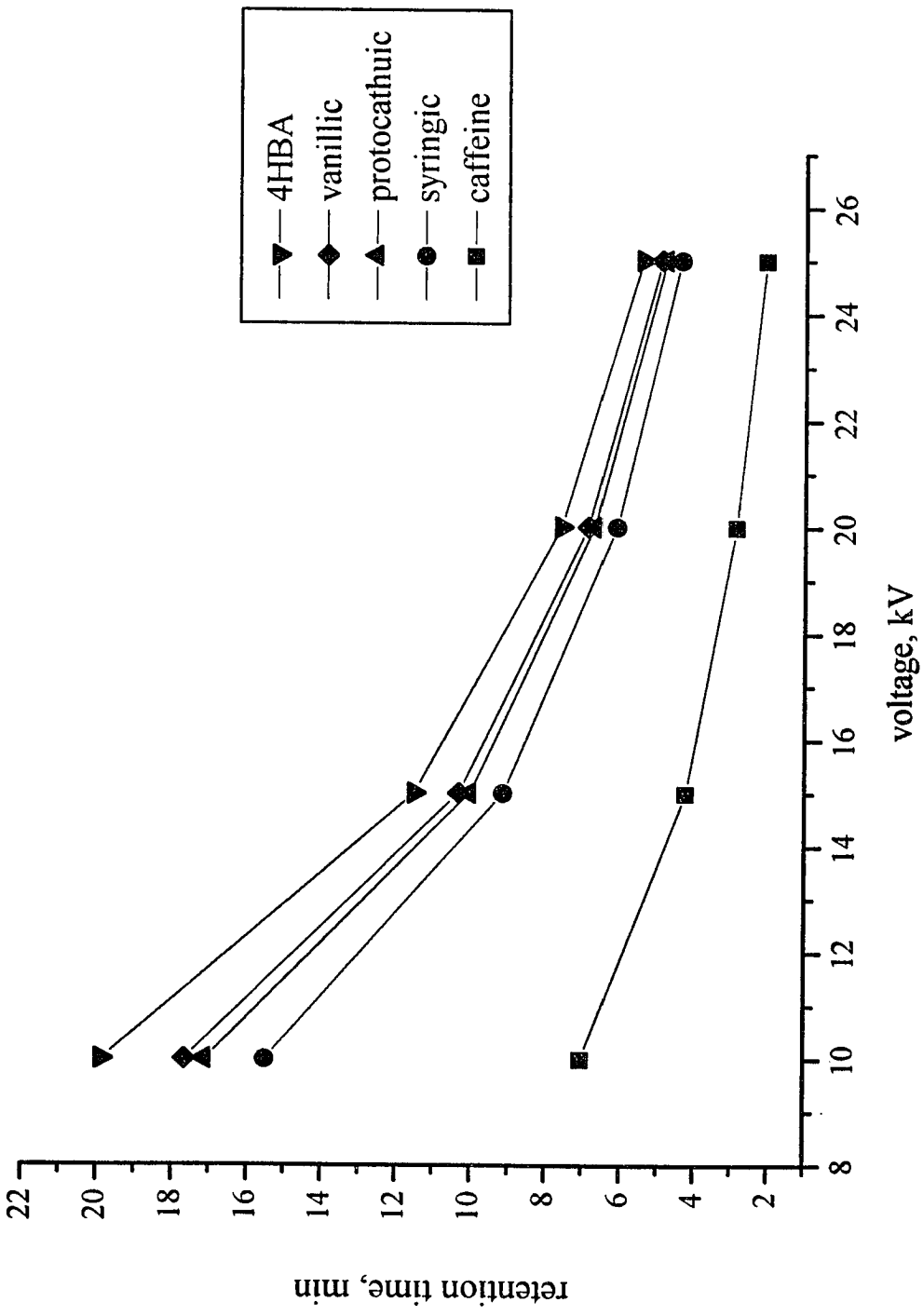


Figure 2-4. Effect of Buffer pH on Retention Times of Organic Acids and Caffeine

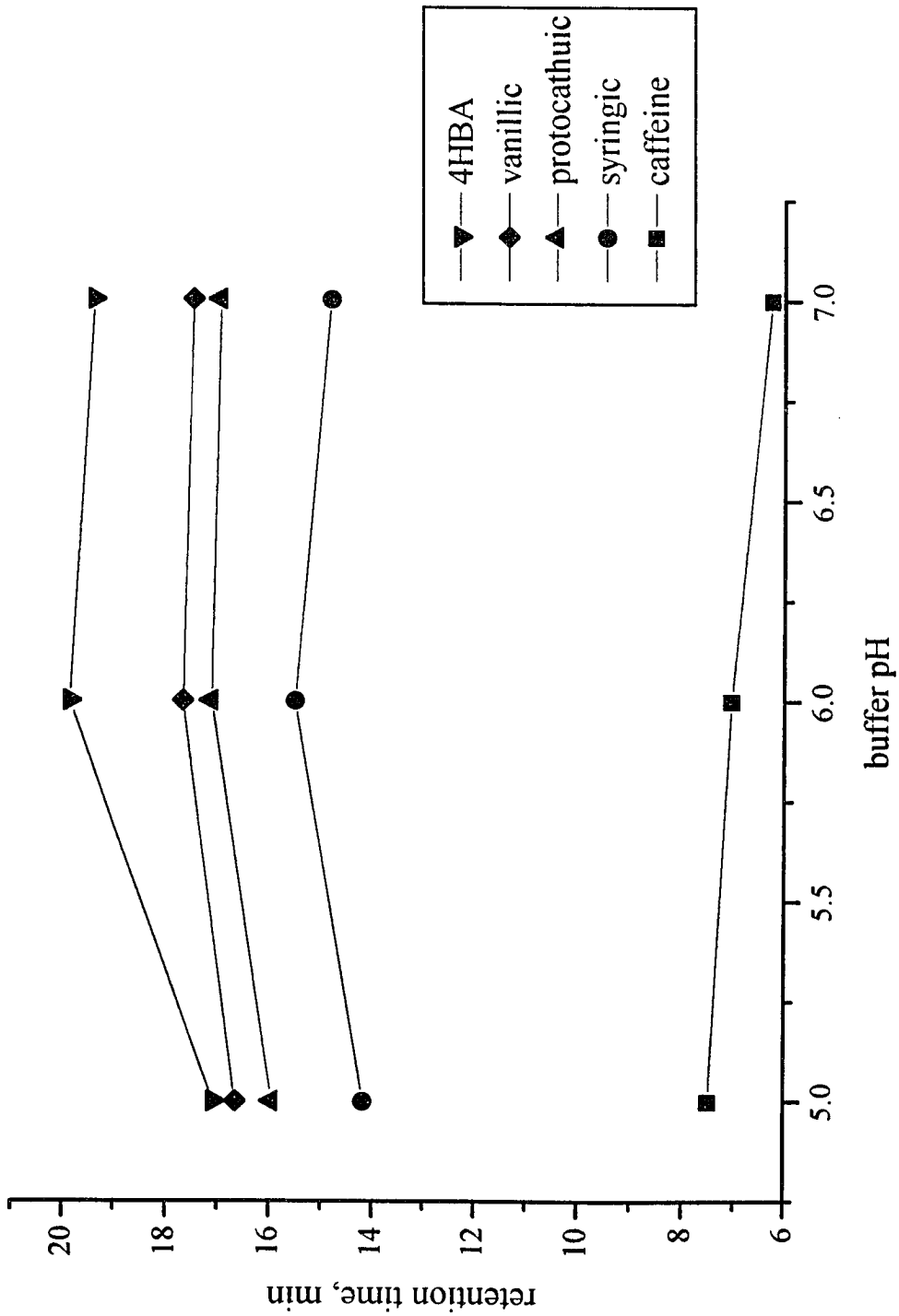


Figure 2-5. Separation of Organic Acids and Caffeine Using a Hydrodynamic Injection.
Peak Identifications: 1)caffeine, 2) syringic acid, 3) protocathuic acid, 4) vanillic acid, 5) 4-hydroxybenzoic acid.

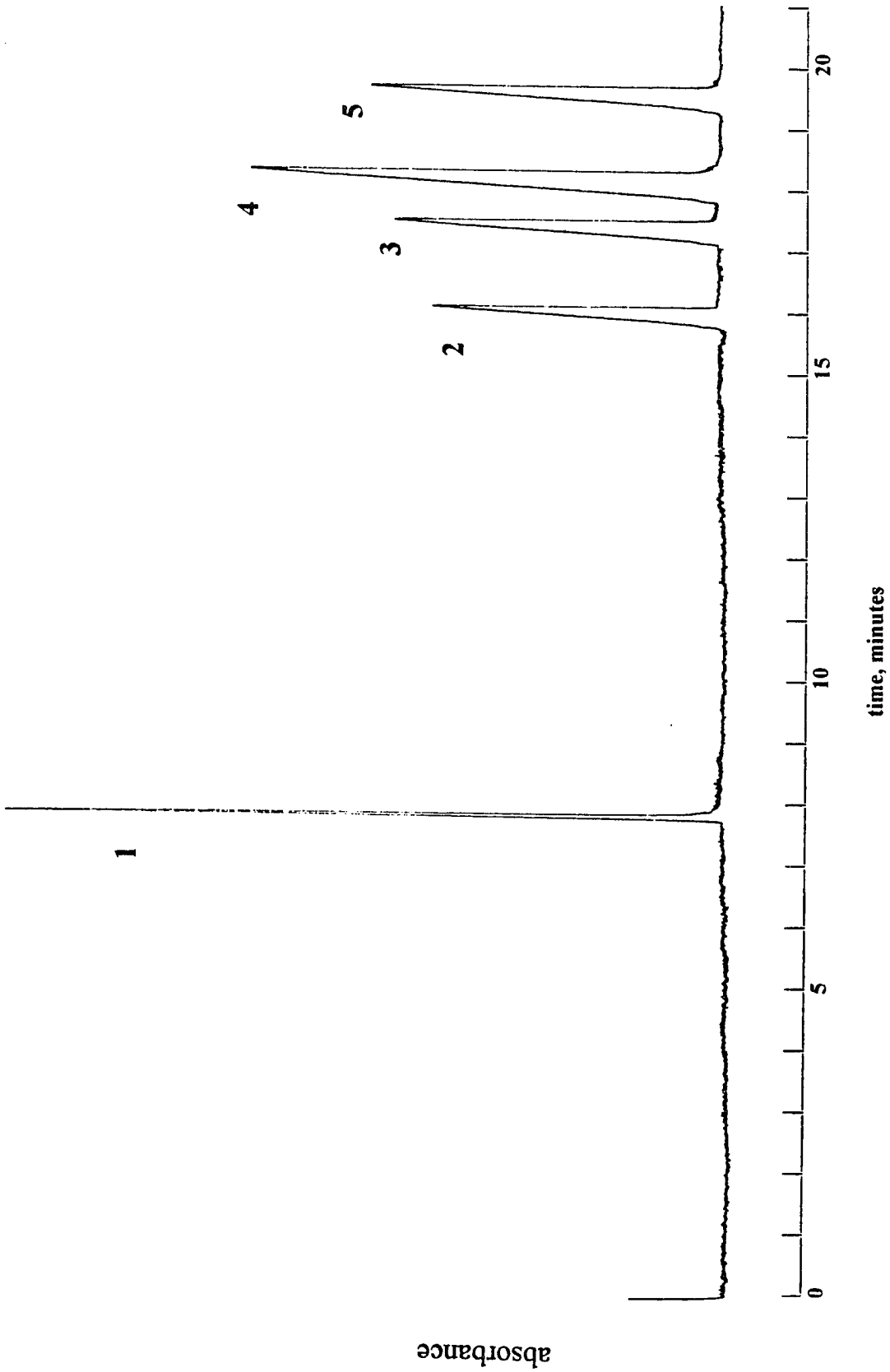
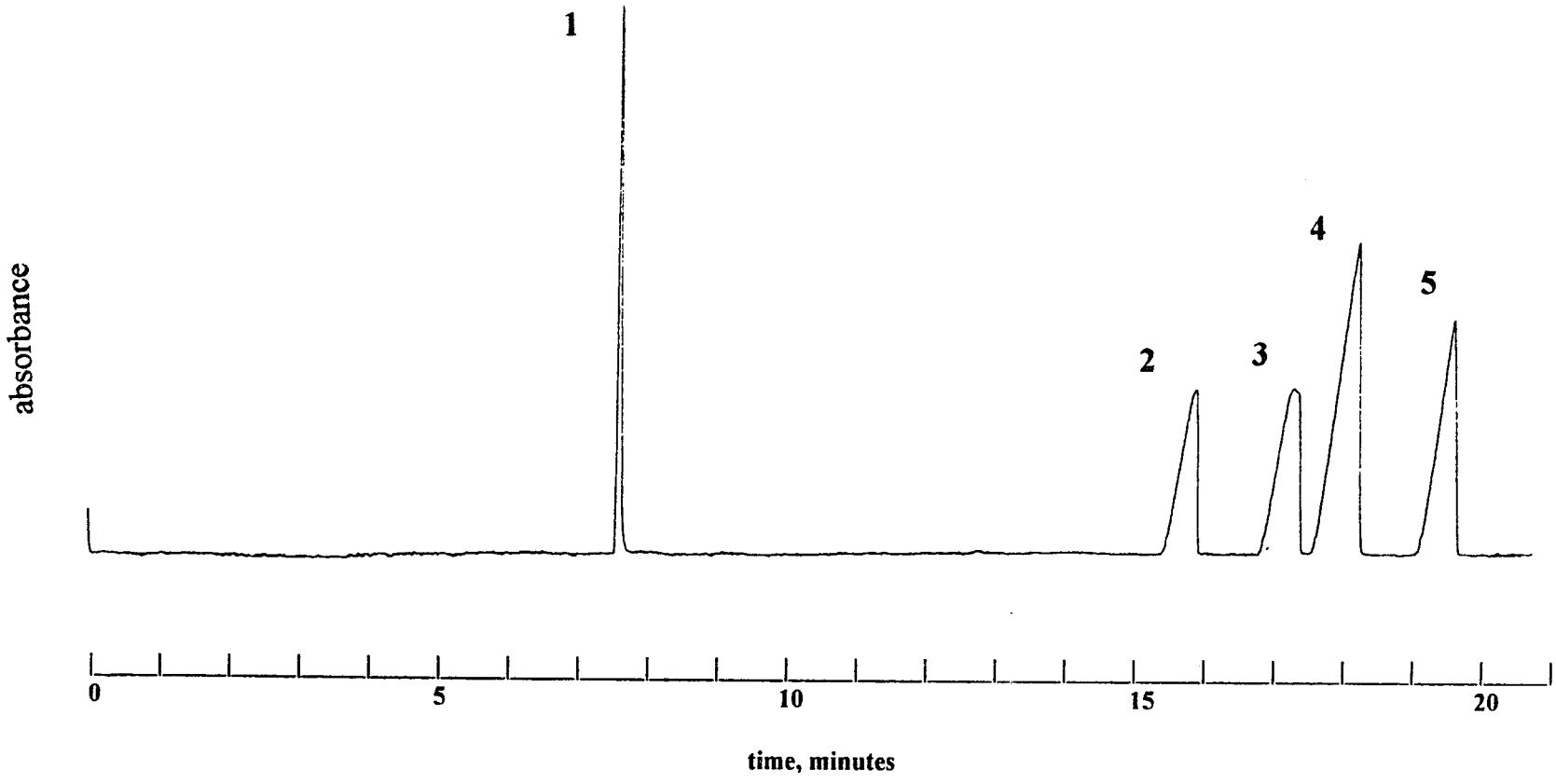


Figure 2-6. Separation of Organic Acids and Caffeine Using an Electrokinetic Injection.
Peak Identifications: 1)caffeine, 2) syringic acid, 3) protocathuic acid, 4) vanillic acid, 5) 4-hydroxybenzoic acid.



CHAPTER 3

COATED CAPILLARIES

3.1. Introduction

Problems can be encountered when using fused silica tubing with an uncoated inner surface for CE separations. The problems include the possibility of poor reproducibility of electroosmotic flow and the adsorption of charged molecules on the capillary surface (1,2). The electrostatic wall-analyte interactions can cause peak tailing and reduce separation efficiency. One way to avoid such problems is to deactivate the silica surface by chemical modification. A theoretical explanation for the use of a polymer coating to eliminate electroosmosis has been provided (3). The effects of electrophoresis and electroosmosis on the migration velocity are presented in equations 1.1 and 1.2. It is possible to suppress electroosmosis by operating under conditions such that $|\mu_{eo}| \ll |\mu_{ep}|$. One way to achieve this is to use materials which are do not attract ions, in which case electroosmosis cannot occur. Several types of polymer coatings have been investigated for this purpose, some of the more successful coatings include polyacrylamide (3,4), polyethylene glycol (5,6), and polyethyleneimine (7).

The work discussed here involves the application of a novel polymeric material produced by Cohesive Biotechnologies (Acton, MA) as a coating material for fused silica capillaries for CE. The polymeric material investigated was a copolymer containing the N-(1-pyridinio) amidate functionality and a vinyl azlactone. Molecules containing an N-(1-pyridinio) amidate functionality have been observed to undergo a photochemical rearrangement to a N-acyldiazepine and fragmentation to generate a very reactive nitrene

species when exposed to uv light at 254 nm. The N-(1-pyridinio) amidate functionality is a very hydrophilic, ionic group whereas the N-acyldiazepine is markedly less polar. The copolymer containing this N-acyldiazepine undergoes rearrangement through the generation of the nitrene species (8). It was the goal of this research to use the photochemical rearrangement to immobilize this polymeric material on the interior surface of the CE capillaries. This coated surface could be further functionalized to provide a desired reduction in electroosmotic flow and reduce adsorption of analytes. Two different chemical functionalizations were used to attempt various reductions in electroosmotic flow and reduced interactions for different types of compounds. The two functionalizations attempted with the polymer coated capillaries were ethanolamine and dimethyloctadecylchlorosilane.

The initial evaluation of the polymer coating was performed by placing the polymer on a potassium bromide crystal. This substrate allowed infrared absorbance data to be obtained for the initial polymer coating, the polymer coating after irradiation with uv light, and the irradiated polymer coating after functionalization. Irradiation of the interior surface of the capillary was attempted using a capillary with an external coating which was stated by the manufacturer (Polymicro Technologies, Inc., Phoenix, AZ) as being transparent in the uv. The first procedure used for coating capillaries were conducted as follows. The capillary tube was filled with a concentrated solution of the polymer for several hours. The polymer solution was removed and the capillary was irradiated for several hours. The capillary was further functionalized as follows. The capillary tube was filled with ethanolamine for several hours, then rinsed with methylene chloride and dried with nitrogen. The second method used to coat the capillaries was a similar procedure, except the capillary was filled with the polymer

solution during the irradiation process. Coated and uncoated capillaries were tested for reduction in electroosmotic flow by capillary zone electrophoresis after being coated with the polymer. Coated capillary tubing was also supplied by Cohesive Biotechnologies and evaluated for several factors which are important for CE columns, including: reduction in electroosmotic flow in CE, level of uv absorbance due to the coating, evaluation of coating using electron microscopy, and effect on band broadening for several solutes.

3.2. Experimental

3.2.1. Instrumentation

Studies were conducted using a laboratory assembled capillary electrophoresis instrument described in Chapter 2. The capillary was bare fused silica, 50 micron ID, 350 micron OD (Polymicro Technologies, Phoenix, AZ), with a uv transparent external coating. The detection wavelength was set at 210nm. Other detector settings were 0.1 absorbance units full scale and a response time of 0.1 seconds.

The infrared spectra were acquired using a Nicolet G-series 520 bench equipped with an MCT-B detector (Madison, WI). The spectra were acquired after coating the polymer on a KBr plate (25mm diameter) and 4mm thick. The spectra were ratioed to a background of the KBr plate without a polymer coating. All spectra were acquired at a nominal 2 wavenumber resolution using 100 scans.

The ultraviolet irradiation of the polymeric material was performed using a Rayonet Photochemical Mini-Reactor (model RMR-500, Southern New England Ultraviolet Co., Hamden, CT). This unit is equipped with four uv lamps each rated for 8 watts at 253.7 nm.

Scanning electron microscopy was conducted using the Amray scanning electron

microscope (model AMR-1000A, Boston, MA) at the University Instrumentation Center Electronmicroscope Facility with the assistance of Ms. Nancy Cherim.

3.2.2. Materials

Reagent grade methylene chloride, acetone, methanol, sodium phosphate and potassium phosphate were obtained from Fisher Scientific. Dimethyloctadecylchlorosilane was obtained from Petrarchsystem, Inc. (Bristol, PA). Ethanolamine and salicylic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bovine serum albumin and DL-histidine were obtained from Sigma Chemical Co. (St. Louis, MO). Dry copolymer, PS-3-049, was supplied by Cohesive Biotechnologies (Acton, MA).

3.2.3. Coating Methods

Initial Evaluation. Dry copolymer PS-3-049, 0.1 g, was dissolved in dry methylene chloride, 0.3 mL, and 10 drops were placed on a cleaned and polished 2.5 cm potassium bromide crystal. Excess methylene chloride was removed with dry nitrogen. A light yellow film was observed on the surface of the plate. The plate was placed in the infrared instrument and a transmission spectrum was collected from 400 to 4000 wavenumbers. The transmission spectrum obtained is given in Figure 1. The plate was then irradiated in the photochemical reactor for 1 hr . A darker yellow film was observed. The transmission spectrum obtained after 1 hr of irradiation is given in Figure 2. The plate was further irradiated for 1 hr. The darker yellow color remained unchanged. The transmission spectrum obtained after 2 hrs of irradiation is given in Figure 3. Three drops of ethanolamine were placed on the polymer surface and allowed to react for 1 hr. Excess ethanolamine was removed with methylene chloride and the film was dried with nitrogen. The transmission spectrum obtained after

treatment with ethanolamine is given in Figure 4. For comparison, Figures 3 and 4 are shown on the same scale in Figure 5.

Capillary Coating. Fused silica capillary tubing, 50 microns internal diameter and 350 microns external diameter, having a uv-transparent external coating was obtained from Polymicro Technologies (Phoenix, AZ). The coating method involved several steps: 1) Filling the capillary with a solution of the copolymer PS-3-049, 0.5 g/mL, in methylene chloride for 2 hrs. 2) Draining the solution from the capillary and drying the inside surface of the capillary with nitrogen. 3) Irradiating for 4 hrs in the photochemical reactor. 4) Filling the coated capillary with ethanolamine for 2 hrs. and 5) Draining the solution from the capillary and drying the inside surface of the capillary with nitrogen. Three attempts were made using this method on capillaries each having a length of 30.6 cm to detection and 39 cm overall. The method was further modified to combine steps 1 and 3 into a single step , by irradiating the capillaries while filled with the copolymer solution. Three more capillaries of the same lengths were treated using this modified method.

3.2.4. Testing of Coated Capillaries As CE Columns.

The testing method involved installing the capillary in the CE instrument and flushing with phosphate buffer for 1 hr at low pressure. The phosphate buffer was prepared at a pH of 7.5 and an ionic strength of 0.01 (9). Electrophoresis was conducted at 10 kV and the migration time of a neutral marker, acetone, was recorded. Electrophoresis was conducted under these conditions using a clear coated fused silica column without any polymer coating as a blank. Migration times for a neutral marker, acetone, were recorded at 2.62, 2.63, and 2.65 min.

Laboratory Coated Columns. Two of the first batch of three columns were successfully installed in the instrument and retention time data were collected for a the neutral marker, acetone, using the same phosphate buffer and voltage previously described. Migration times for duplicate analyses were recorded at 2.62 and 2.64 for the first column and 2.65 and 2.67 for the second column. One of the columns using the second coating method was successfully installed in the instrument and retention time data were collected for a neutral marker, acetone, using the same phosphate buffer and voltage as before. Migration times for duplicate analyses were recorded at 2.62 and 2.71 for this column.

Polymer Coated Columns Supplied by Cohesive Biotechnologies. A 3 m length of the clear-coated column was supplied already coated with the copolymer. A 39 cm piece of this column was treated with ethanolamine for 2 hr, rinsed with methylene chloride, and dried with nitrogen. Another 39 cm piece of this column was treated with a solution of dimethyloctadecylchlorosilane, 0.5 g/mL, for 6 hr, rinsed with methylene chloride and dried with nitrogen.

The polymer coated column and the coated columns treated with ethanolamine and dimethyloctadecylchlorosilane were tested using the same procedure which was used for the laboratory coated columns. A 39 cm piece of each of these columns was installed in the CE instrument with 30.6 cm to detection. Electropherograms showing the migration times of the neutral markers acetone and methanol are given in Figures 6 and 7, respectively. The retention times observed and theoretical plates calculated for the test analytes for bare fused silica, polymer coated, polymer coated and functionalized with ethanolamine, and polymer coated and functionalized with dimethyloctadecylchlorosilane capillaries are given in Table

1. The compounds investigated include acetone, methanol, benzoin, bi-2-naphthol and bovine serum albumin.

3.2.5. Absorbance of Capillary Coating

The absorbance of the polymer coated capillary versus the uncoated capillary is given in Figure 8 for air filled and buffer filled capillary. These measurements were conducted by: 1) Recording the absorbance for the bare fused silica capillary when filled with air and buffer, 2) Recording the absorbance for the polymer coated fused silica supplied by Cohesive Biotechnologies when filled with air and buffer, and 3) Subtracting the absorbance for bare fused silica capillaries from the coated capillaries. The detection wavelength was adjusted to 200 nm and autozeroed. The desired wavelength was then set on the detector and the absorbance recorded. Measurements were recorded at 5 nm intervals between 200 and 300 nm, and at 10 nm intervals between 300 and 400 nm.

3.2.6. Electron Microscopic Investigations of Capillary Coatings.

The polymer coating on the interior surface of the capillaries supplied by Cohesive Biotechnologies was investigated using scanning electronmicroscopy. The electronmicrographs obtained for the capillary end at magnifications of 230X and 1200X are given in Figures 9a and 9b, respectively. Capillaries were then carefully micro-manipulated to produce a lengthwise split in the fused silica. The electronmicrographs obtained for the internal surface of coated and uncoated split capillaries are given in Figure 10a and 10b, respectively.

3.3. Results and Discussion

Initial studies conducted on the salt plate provided the first evidence of the polymer

coating. Visual observations and spectroscopic evidence presented in Figures 1-4 supported the coating of the polymer and the functionalization with ethanolamine. No significant change in the infrared spectra were observed after 1 and 2 hours of irradiation in Figures 2 and 3. These data indicated the completion of the photochemical reaction in 1 hour using direct irradiation. The disappearance of the band at 1810 wavenumbers was observed in the spectrum presented in Figure 4 . The disappearance of this band indicated that a reaction had occurred on the surface of the polymer after treatment with ethanolamine. This evidence supported the functionalization of the polymer with ethanolamine (8).

The retention time data obtained using capillaries which were coated in our laboratory did not indicate significant reductions in the level of electroosmotic flow. It was clear that the polymer coating was not adhering to the capillary surface using the coating techniques described here. The major consideration in immobilization of the polymeric material was the uv irradiation. The most likely reason for our failure to immobilize the polymer on the surface of the capillary was that the intensity of uv light reaching the interior of the capillary was insufficient to cause the photochemical rearrangement to occur.

Using polymer coated capillaries supplied by Cohesive Biotechnologies results were obtained that were consistent with having a polymer coating on the interior surface of the capillaries. The capillary electrophoresis data are given in Table 1. Figures 6 and 7 are electropherograms which show a reduction in electroosmotic flow using the neutral markers and an increase in theoretical plates for the selected compounds. The acids would be expected, as charged molecules, to be affected by both electroosmosis and electrophoresis, thus their migration times would be expected to be longer in CZE than the migration times

of neutral compounds. The data obtained show the protein, albumin, is still affected by wall interactions which cause peak broadening, but some improvement in efficiency is observed.

Further evidence to support the presence of the polymer coating on the interior surface of the capillary is given by the increase in uv absorbance of the coated capillary as shown in Figure 8. The polymer coating has been reported to have an absorbance in the 200 to 400 nm range (8).

The electron microscopy images presented in Figure 9 show a clear picture of the capillary tip, but it is difficult to see the interior clearly. The split capillaries show a film on the surface in Figure 10a with the edge of the film observable in the bottom of the image. The bare surface with some particulate matter is observed in Figure 10b.

3.4. Conclusions

The biggest challenge in testing the capillaries was the brittle nature of the clear-coated capillaries. Once the capillary end has been in contact with water for more than 1 hr, the external coating begins to separate from the fused silica. The weakened coating comes off the ends of the column when placed under any mechanical stress. This results in an uncoated piece of the column for the first 1 to 10 cm and the brittle fused silica breaks rapidly in our instrument.

Some promising results towards the limiting of electroosmotic flow and solute interaction are observed using this polymer coating. If a rugged external coating which is transparent in the ultraviolet is developed, this may be prove to be an excellent method of preparing coated CE columns.

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Table 3-1. Retention Times and Theoretical Plates Observed for Several Compounds with Different Columns. Capillary Electrophoresis conducted at 10kV using a phosphate buffer of pH = 7.5 and I = 0.01. Theoretical Plates, N, were calculated from peak widths at half height and migration times for each solute.

	bare fused silica		polymer coated		polymer coated and functionalized with ethanolamine		polymer coated and functionalized with dimethyloctadecyl-chlorosilane	
	t_R	N	t_R	N	t_R	N	t_R	N
acetone	2.68	4000	12.57	3500	4.18	6700	4.63	18100
methanol	2.63	6300	11.96	3450	5.19	10300	5.88	6900
salicylic acid	5.88	400	15.51	12000	8.49	15400	10.05	17400
DL-histidine	5.55	8100	23.10	16000	7.98	14500	9.95	16300
albumin	7.65	500	52.10	2000	8.23	4300	22.10	5200

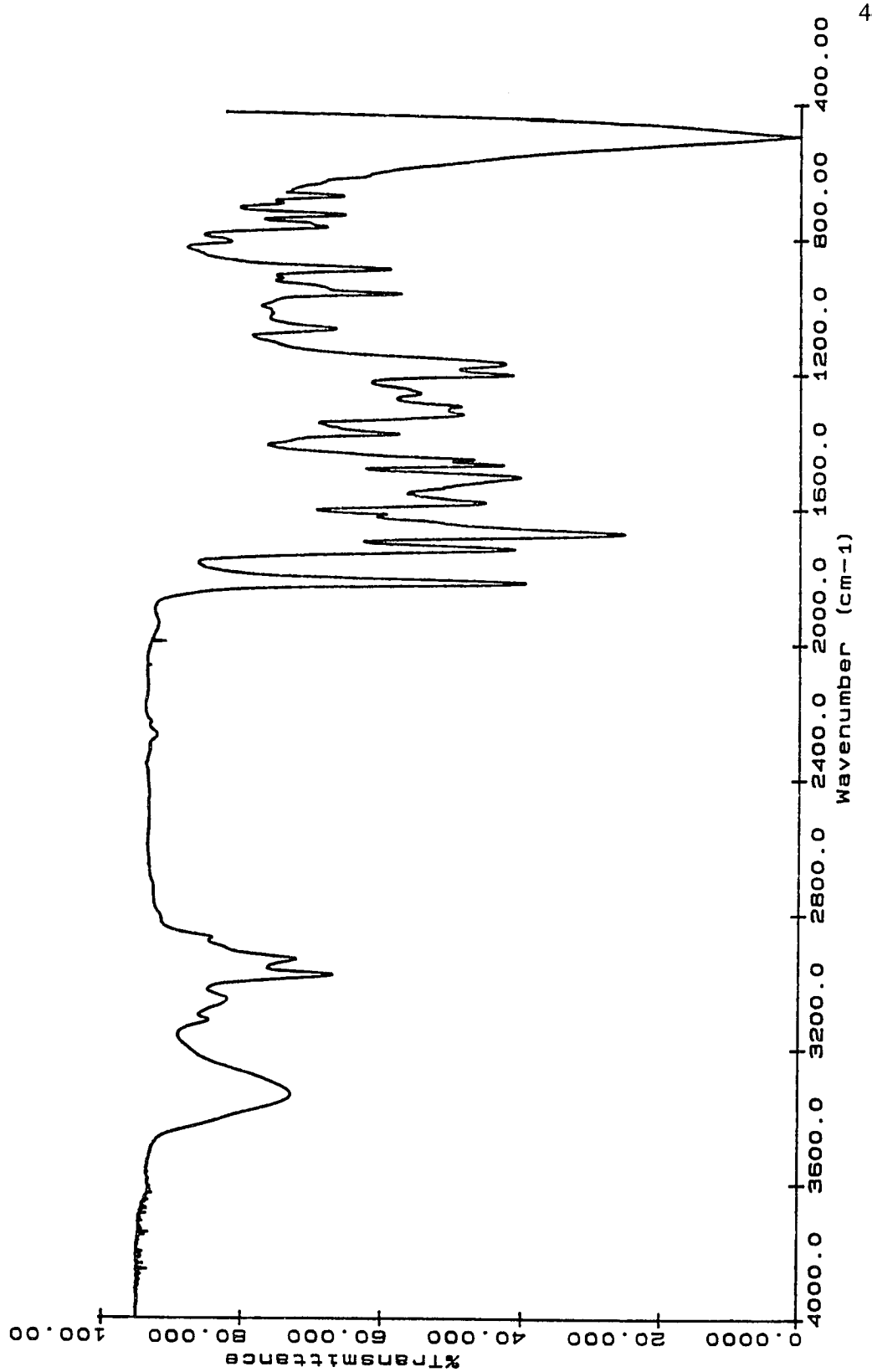


Figure 3-1. Infrared Spectrum of Polymer Coating on KBr Plate Before Irradiation

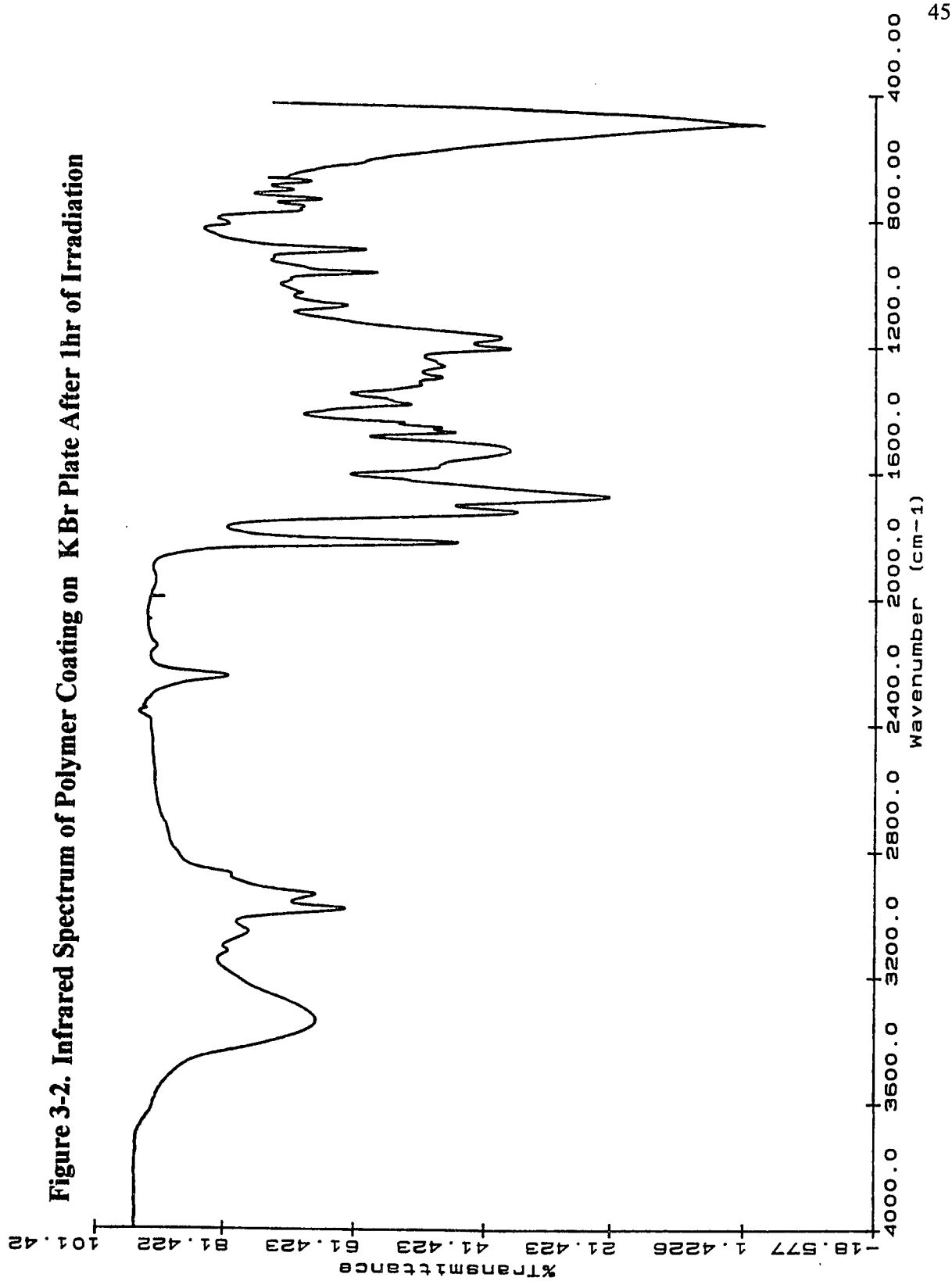


Figure 3-2. Infrared Spectrum of Polymer Coating on KBr Plate After 1hr of Irradiation

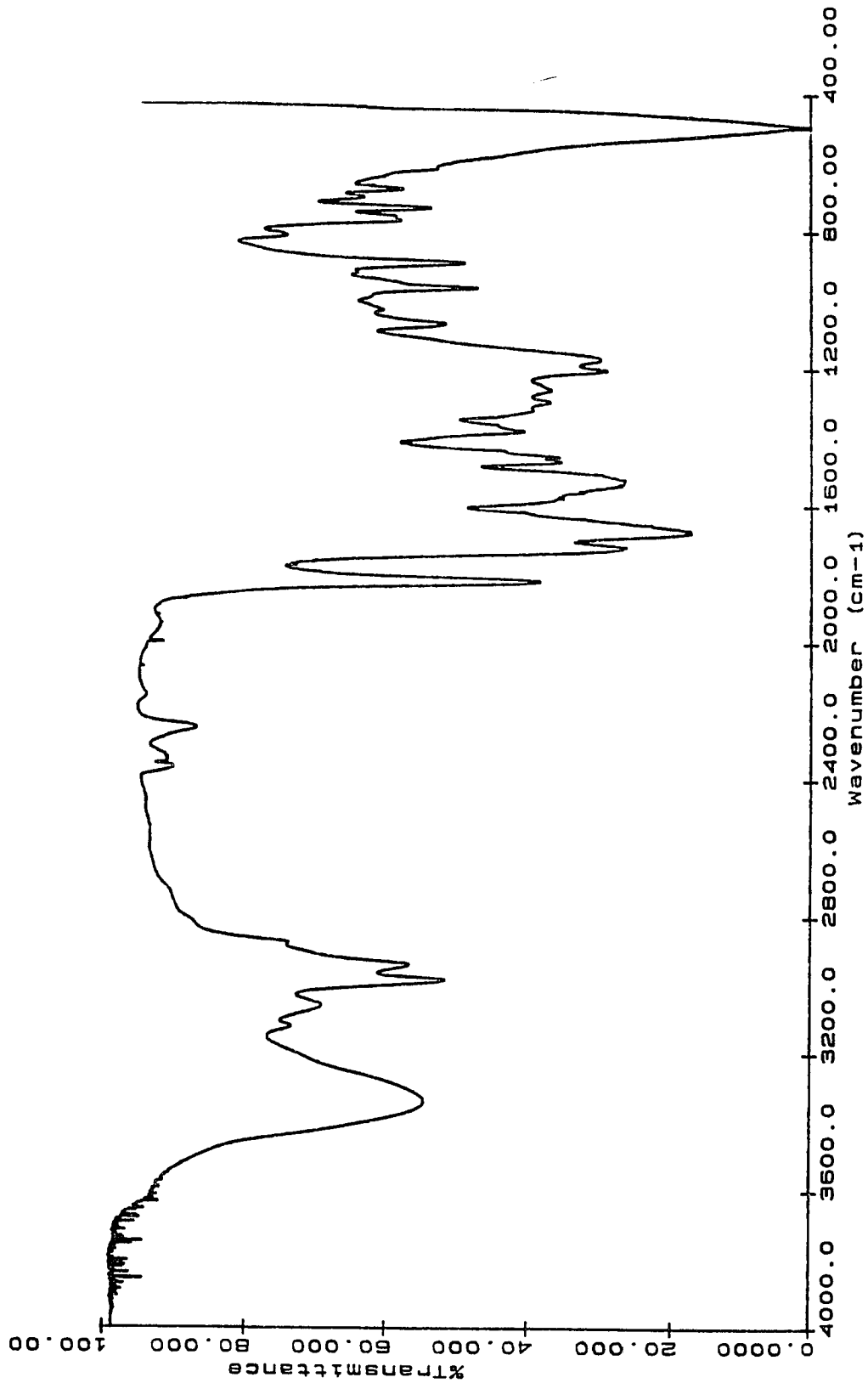


Figure 3-3. Infrared Spectrum of Polymer Coating on KBr Plate After 2hr of Irradiation

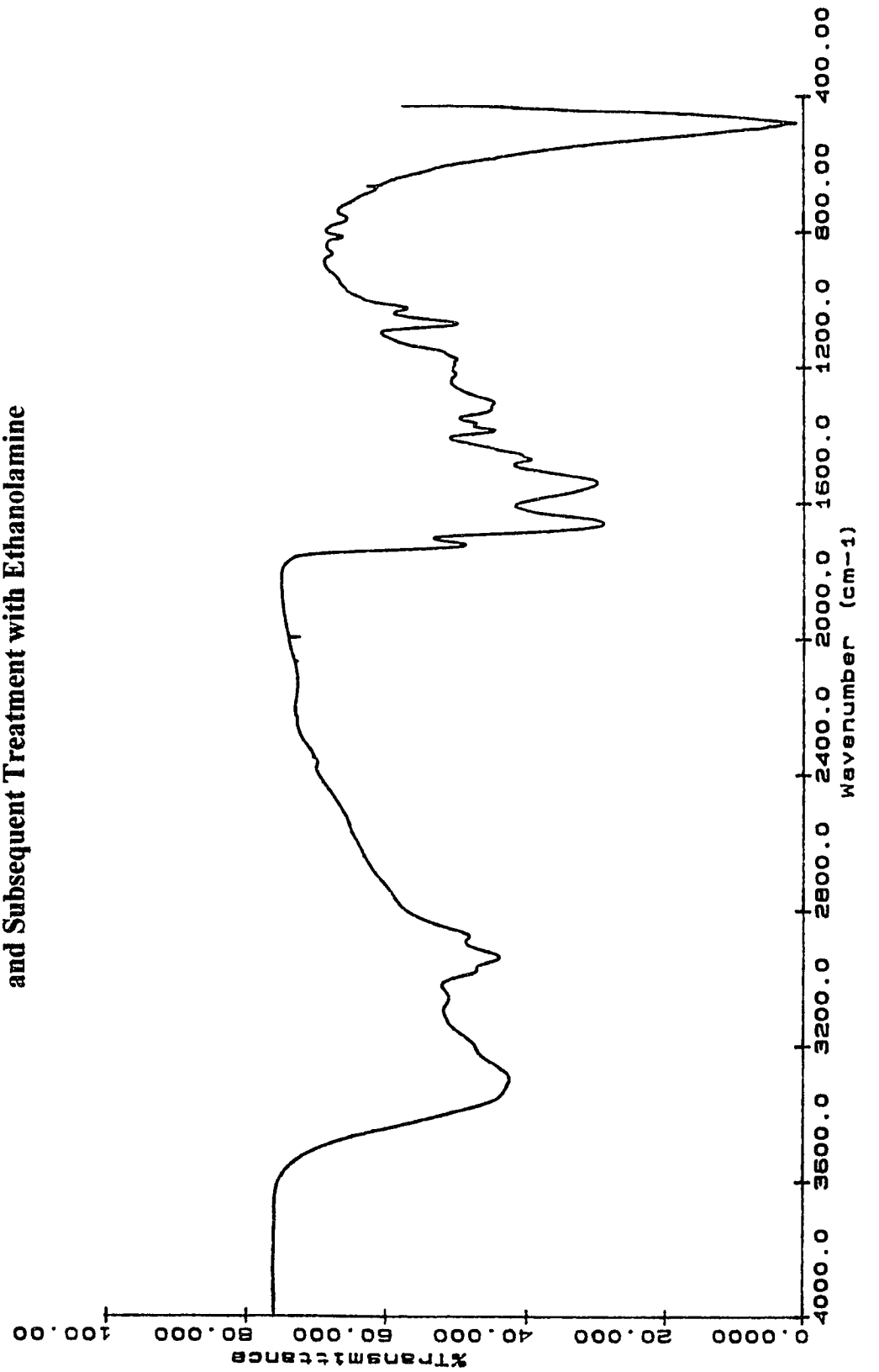


Figure 3-4. Infrared Spectrum of Polymer Coating on KBr Plate After 2hr of Irradiation and Subsequent Treatment with Ethanolamine

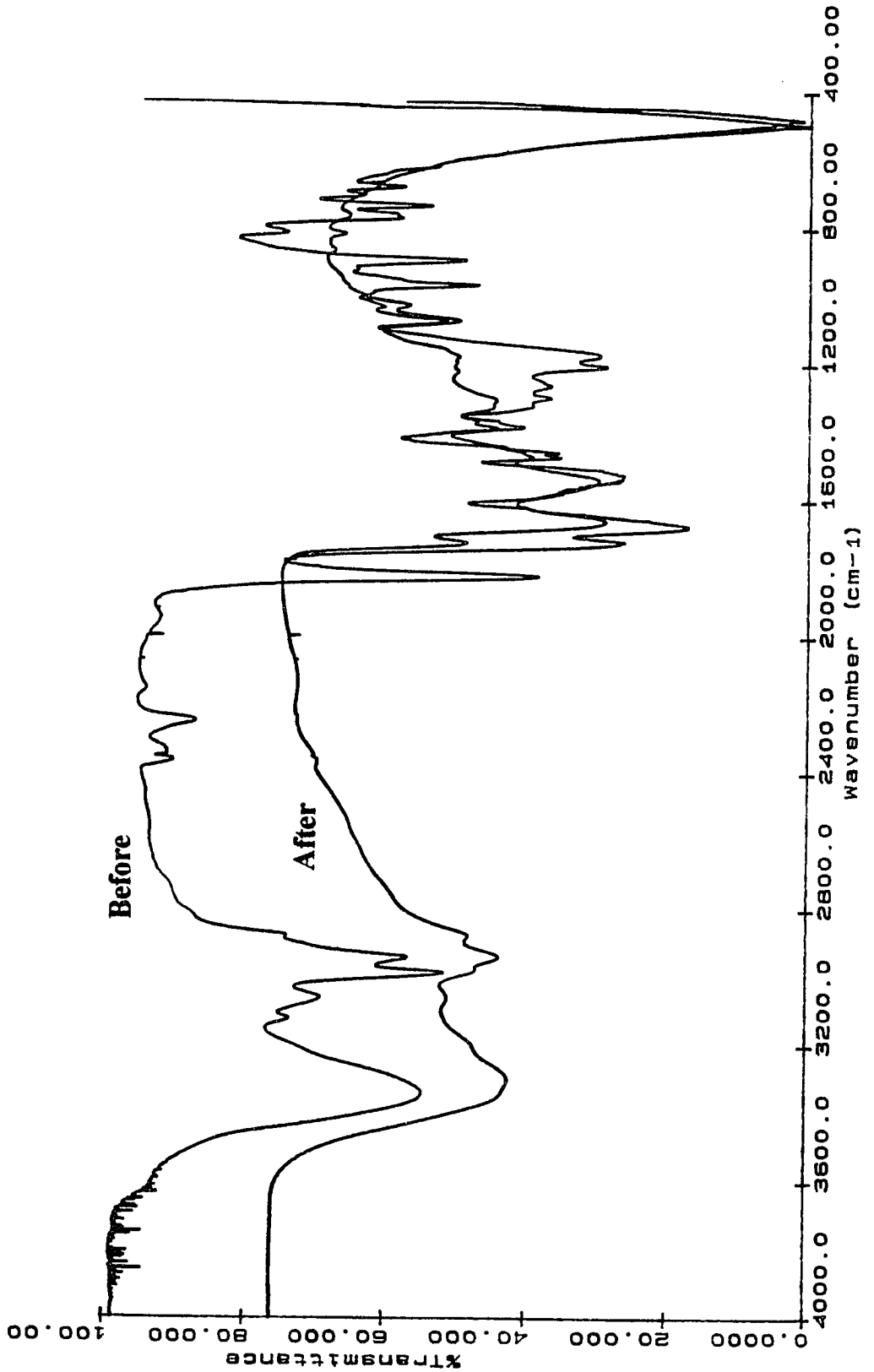


Figure 3-5. Comparison of Infrared Spectra Before and After Treatment with Ethanolamine

Figure 3-6. Migration of Acetone Using Bare Fused Silica and Polymer Coated Columns in Capillary Electrophoresis

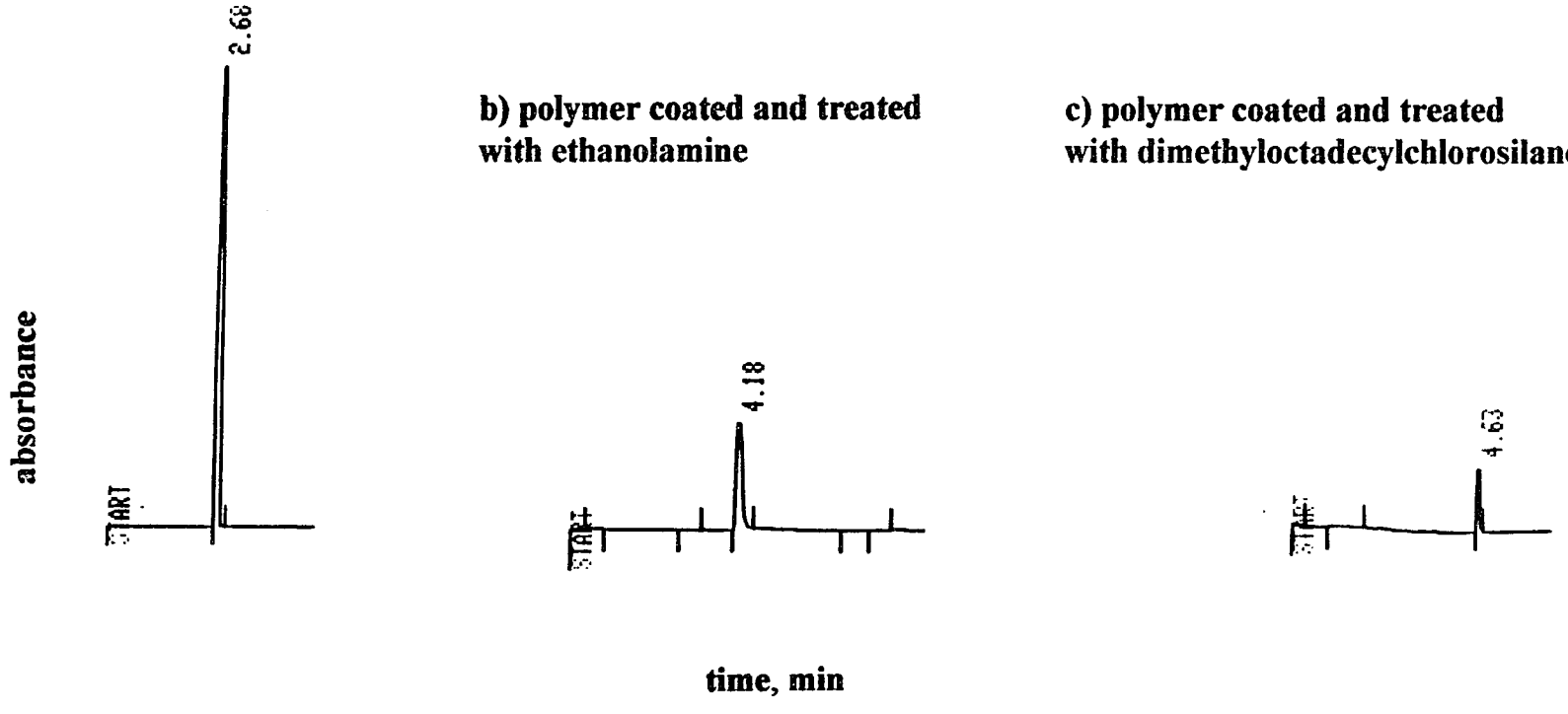


Figure 3-7. Migration of Methanol Using Bare Fused Silica and Polymer Coated Columns in Capillary Electrophoresis

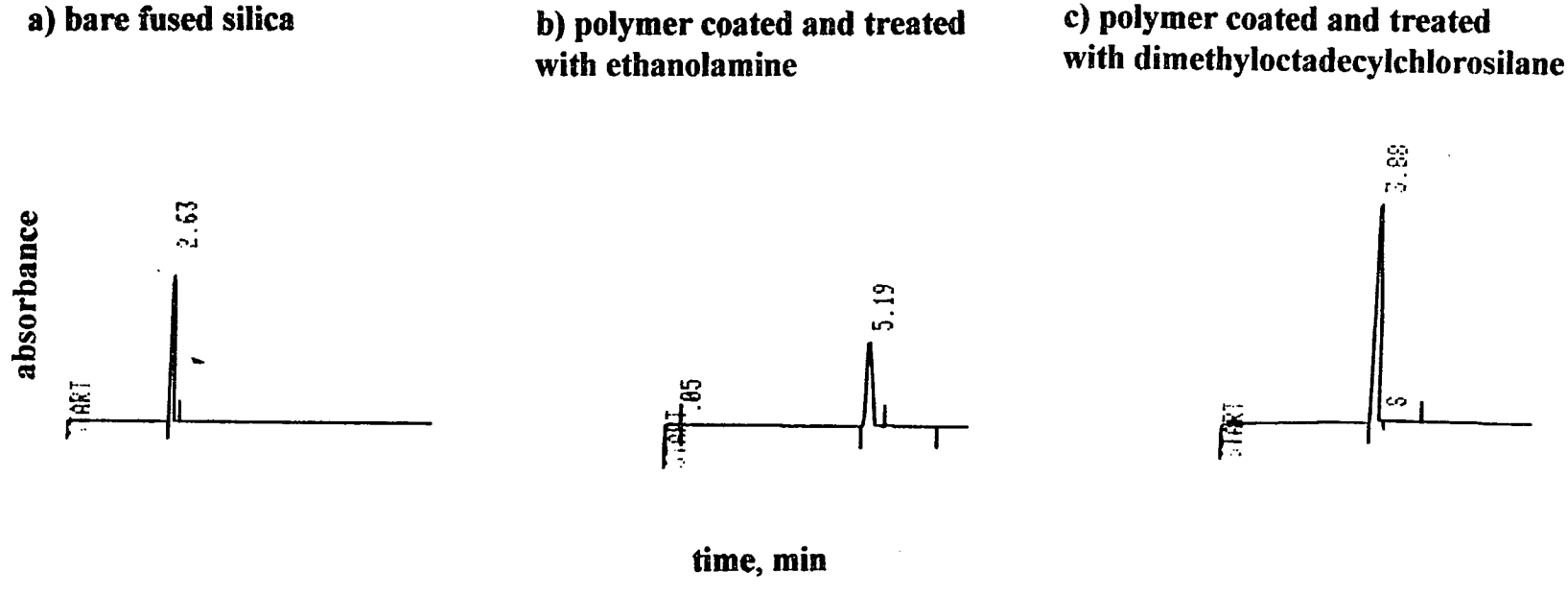


Figure 3-8. Absorbance Increase of Coated Columns for capillaries containing air or phosphate buffer

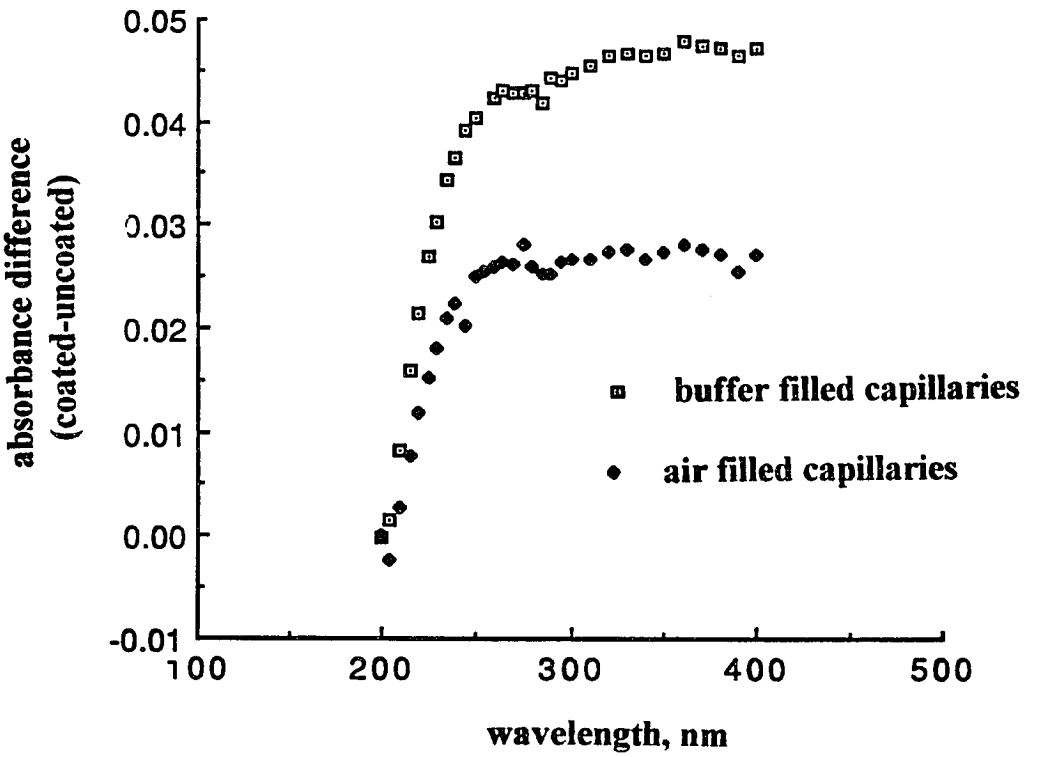
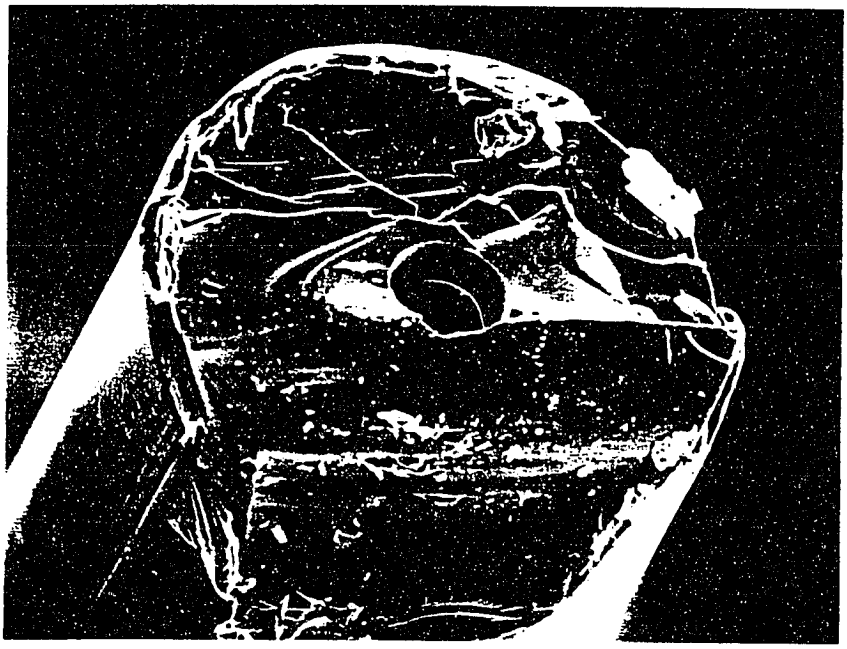
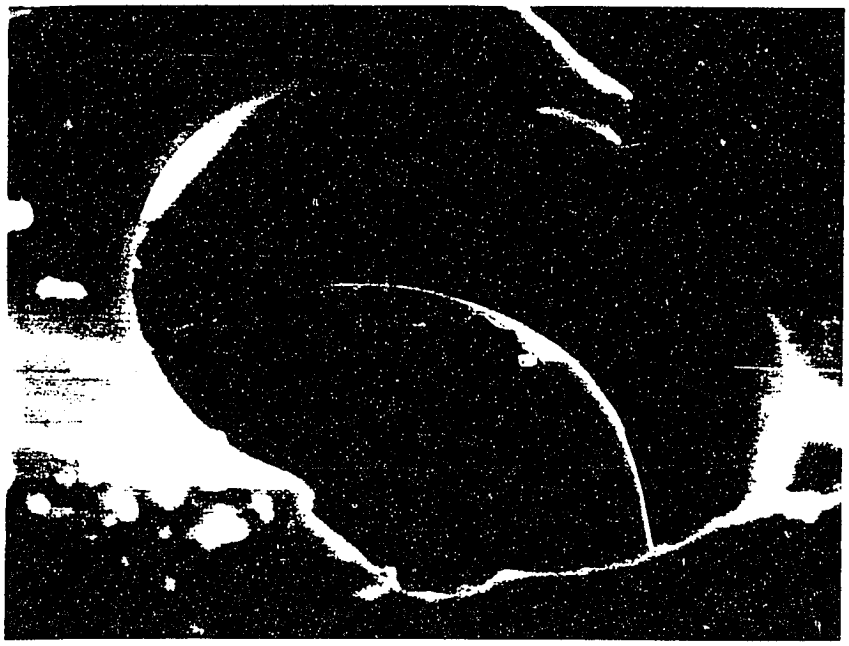


Figure 3-9. Electronmicrographs of a Fused Silica Capillary

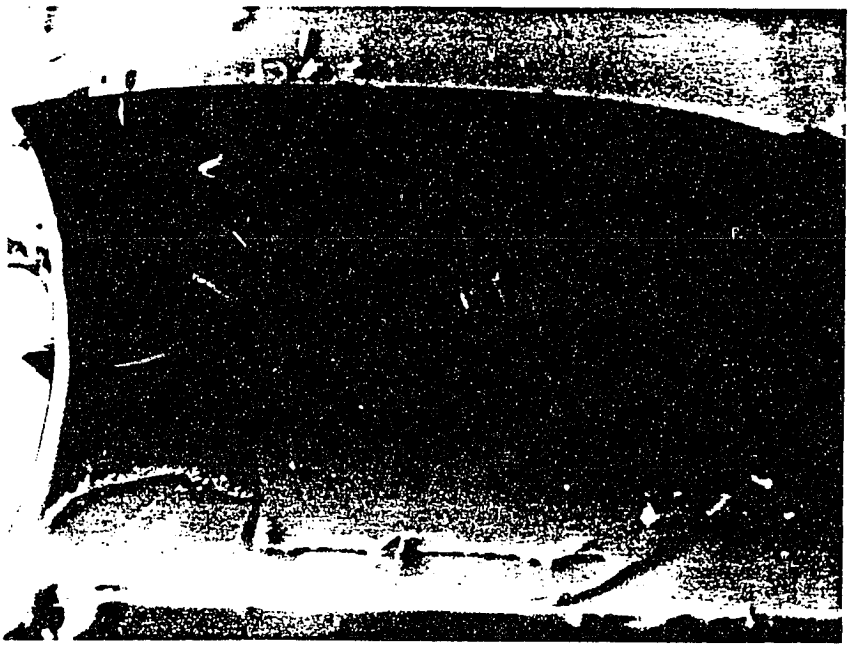


a) Capillary End at a Magnification of 230X

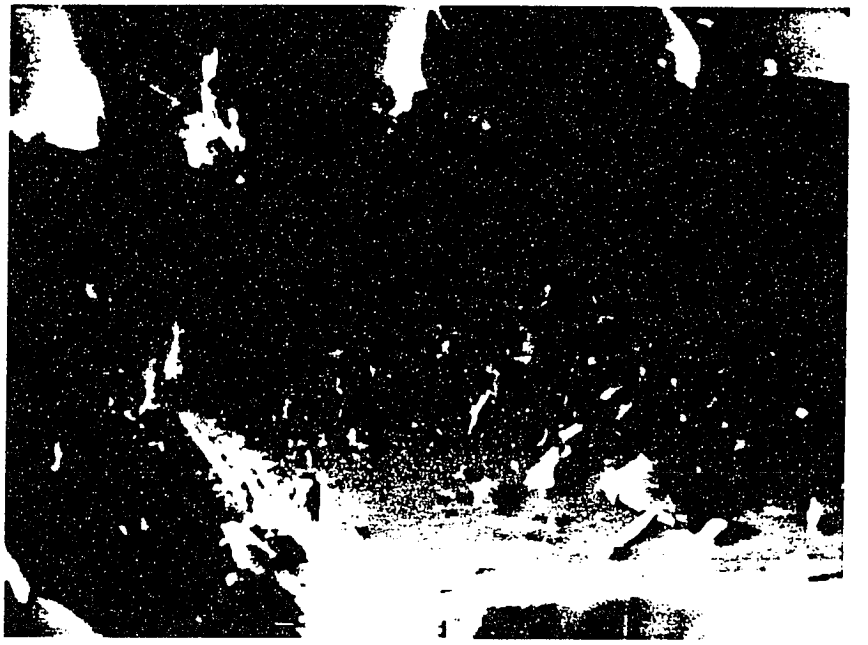


b) Capillary End at a Magnification of 1200X

Figure 3-10. Electronmicrographs of a Fused Silica Capillary



a) Inside Surface of Coated Column at a Magnification of 1000X



b) Inside Surface of Uncoated Column at a Magnification of 1000X

CHAPTER 4

USING BILE SALTS AS CHIRAL PSEUDOSTATIONARY PHASES IN CE

4.1. Introduction

Bile salts are naturally occurring chiral surfactants which have been widely studied due to their physiological importance and unique chemical and physical properties. The primary physiological role of these compounds is to solubilize fats during the digestion process. Many forms of the bile salts are found in nature. The class of compounds known as the "bile salts" have a common sterol backbone but differ in the number, position and orientation of the hydroxyl groups (1). Two bile acids, cholic acid and chenodeoxycholic acid, are synthesized from cholesterol in the human liver (2). Once synthesized they are conjugated with the amino acids taurine or glycine. Other bile acids are formed by the action of intestinal microorganisms. Normal human bile contains 50% of the trihydroxy bile salt epimer, cholate, 15% and 30% of the dihydroxy bile salt epimers, deoxycholate and chenodeoxycholate, respectively, and 5% of the monohydroxy epimer, lithocholate(3). These numbers are approximate due to the significant effect which diet has on the relative ratios.

The importance of the bile salts as naturally occurring surfactants has resulted in many studies concentrating on determining the physical properties and fundamental constants (critical micelle concentration, micellar aggregation number, solution surface tension, and viscosity) of aqueous bile salt solutions. A review of the literature concerning the determination of size and aggregation number of bile salt micelles in aqueous solutions using static light scattering, sedimentation equilibrium, membrane osmometry, sedimentation velocity, and translational diffusion techniques indicates there is a great deal of variability in

the reported results (4). The scatter in the data was found to be related to the precise control of solution and environmental variables. Both salt concentration and temperature were shown to greatly influence the reported results. Careful control of experimental conditions is of the utmost importance in obtaining a bile salt solution with reproducible aggregation properties. Another method reported for the characterization of bile salt aggregates in aqueous solutions is NMR spectroscopy (5,6). Proton and Carbon-13 NMR studies were used to characterize bile salt solutions and provide data to support models of bile salt aggregation. Changes in the aggregation properties were correlated to the concentration of various metal ions and micellar solution age.

Several reports in the literature indicated that chiral separations are possible using bile salts as mobile phase modifiers in capillary electrophoresis (7-13). The effects of pH and bile salt species were investigated for the chiral separations of carboline derivatives and bi-2-naphthol (8). Chiral separation of dansylated dl-amino acids was also achieved by MECC with neutral and acidic solutions of sodium taurodeoxycholate (7). Chiral resolution was also observed for the enantiomers of diltiazem hydrochloride, trimetoquinol hydrochloride and related compounds using MECC with neutral solutions of sodium taurodeoxycholate (9,10). The effects of methanol as a mobile phase modifier were also investigated for bi-2-naphthol and related compounds when using bile salts solutions in MECC (13).

The examples of bile salt MECC separations in the literature were intriguing, yet limited in that a certain bile salt was only cited as providing a chiral separation for a certain set of similar molecules. A great deal of information is available on the chemical and physical properties of the bile salts. Little or no evidence is given for the behavior of the bile salts

inside the capillary tube during the capillary electrophoresis experiment. The only data available is electrokinetic chromatography data; run times, peak shapes, etc. Most of the authors of the publications reviewed did not try to unravel what was happening, it was simply termed micellar and treated like a normal sodium dodecyl sulfate (SDS) micellar separation. The goal of the studies described here was to determine what effect experimental variables such as bile salt structure, bile salt concentration, co-solvent, and solution pH have on obtaining chiral separations using bile salts as a pseudostationary phase in CE. It was anticipated that the knowledge gained by evaluating systematically the effects of these variables on the separation would allow a better utilization of bile salts as mobile phase modifiers and also provide a better understanding of the mechanism of the bile salt - enantiomer interaction.

The research described here was undertaken in three steps 1) Investigations to determine the instrumental requirements and enhancements necessary to conduct MECC separations with bile salts, 2) characterization of carefully prepared bile salt solutions, and 3)applying these solutions to chiral separations of some of the test compounds previously reported in the literature to separate by MECC with bile salts. The structures of the bile salts used in these studies, sodium taurodeoxycholate and sodium deoxycholate, are given in Figure 4-1. The structures of the solutes investigated, dl-laudanosine and bi-2-naphthol, are given in Figure 4-2. The first step was accomplished by investigating a reported separation of dl-laudanosine using solutions containing sodium taurodeoxycholate (3). These separations allowed the evaluation of the laboratory assembled CE instrument to conduct chiral separations. The second step concentrated on viscosity measurements and NMR spectroscopy

to determine the solution properties of the unconjugated bile salt sodium deoxycholate. The third step involved the evaluation of these NaDC solutions as chiral pseudophases for the separation of the enantiomers of bi-2-naphthol in MECC.

4.2. Experimental

4.2.1. CE Instrument

Studies were conducted using the laboratory assembled capillary electrophoresis instrument using the improvements described in Chapter 2. Experiments were performed using both 50 micron ID bare fused silica capillaries and coated capillaries (Supelco, Bellefonte, PA). The absorbance at 210nm was recorded using both a strip chart recorder (Kipp & Zonen, Holland) and a model 3390A integrator (Hewlett-Packard, Wilmington, DE).

4.2.2. Materials

Sodium deoxycholate (NaDC) and sodium taurodeoxycholate (NaTDC) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The manufacturer stated purities of NaDC and NaTDC were greater than 98% and 95%, respectively. The NaDC was recrystallized from ethanol prior to use (14). The NaTDC was used as received due to the preliminary nature of the investigations. The R and S enantiomers of bi-2-naphthol and a racemic mixture of laudanosine were purchased from Aldrich. The manufacturer-stated purities of the bi-2-naphthol compounds and dl-laudanosine were 99%. The manufacturer stated that the dl-laudanosine was prepared using an achiral synthesis and was an equal mixture of the two enantiomers. Reagent grade sodium phosphate, sodium hydroxide and sodium chloride and HPLC grade methanol and dioxane were purchased from Fisher Scientific (Pittsburgh, PA). Sudan III, a widely used micelle marker, was purchased from

Central Scientific (New York, NY). Deionized and distilled water was used for the preparation of all solutions. Spectral grade D_2O was purchased from Cambridge Isotope Laboratories (Cambridge, MA) and used for the NMR experiments.

4.2.3. Chiral Separation of dl-Laudanosine

Phosphate buffers with an ionic strength of 0.01 were prepared at pH values of 4.2, 6.7 and 7.9. Appropriate amounts of NaCl were added to prepare additional buffer solutions with ionic strengths of 0.05, 0.15 and 0.50. Twelve NaTDC solutions, 50mM, were prepared using these twelve buffer solutions. These twelve solutions were tested as micellar solutions for the separation of dl-laudanosine. The racemic mixture of laudanosine was used to study the separation of the enantiomers. The individual enantiomers of this compound could not be obtained. The manufacturer stated purity of 99% and enantiomeric ratio of 50:50 were used to suggest that a chiral separation was occurring when peaks of equal intensity were obtained in the electropherograms. The electrophoretic data were acquired using a C_8-Bonded Phase column (Supelco, Bellefonte, PA), 34 cm to detection and 45 cm overall, at an applied potential of 10 kV. The solutions with a pH of 4.2 were noticeably more viscous than the solutions at higher pH. No electroosmotic flow was observed using solutions at a pH of 4.2 and they caused the column to clog rapidly. No useable data were collected for the solutions at a pH of 4.2. The current observed by CE was high and unsteady using solutions prepared from buffers at an ionic strength of 0.50 and pH values of 6.7 and 7.9. Electrophoresis could only be conducted using the high ionic strength buffers for 5 minutes or less before the observed current went to zero. A high current was observed using the high ionic strength solutions which probably led to the formation of air bubbles and the drop in current. The

remaining six solutions were tested as chiral MECC pseudophases. A plot of resolution versus ionic strength for the enantiomers of dl-laudanosine using each of these six solutions is given in Figure 4-3. The electropherograms obtained using solutions prepared from buffers at a pH of 6.7 and ionic strengths of 0.05 and 0.15 are given in Figure 4-4. The electropherograms obtained using solutions prepared from buffers at a pH of 7.9 and ionic strengths of 0.01 and 0.15 are given in Figure 4-5.

4.2.4 Viscosity of Bile Salt Solutions

Instrument. The viscosity was measured with a model DV-I+ viscometer (Brookfield Engineering Laboratories, Stoughton, MA). This viscometer was fitted with a small sample adapter which requires as little as 9 mL of solution for a measurement. A thermal jacket for the sample compartment was connected to a water bath which was held at 25° C.

NaDC Solutions. The effect of pH on the viscosity of NaDC solutions was investigated. A 50mM solution of NaDC was prepared and adjusted to seven different pH values in the range of 7.15 to 8.48 using dilute solutions of NaOH and HCl. The viscosities of these solutions were measured using a spindle velocity of 12 rpm. A plot of the observed viscosities of these solutions versus pH is given in Figure 4-6.

The effect of age on viscosity was investigated for a 50mM NaDC solution at a pH of 7.80. A plot of the observed viscosities versus the time since preparation for the solution, up to 14 days, at three different spindle velocities are given in Figure 4-7. The pH of the solution was also monitored at the same time intervals as the viscosity and found to be at 7.80±0.05 throughout the study.

4.2.5. NMR Studies of NaDC Solutions.

^{1}H NMR Studies. Solutions of NaDC were prepared in D_2O at concentrations of 5 and 50mM at a pH of 7.9 and at 50mM at a pH of 10.25. ^{1}H NMR spectra were acquired for these three solutions using a model AM 360 NMR spectrometer (Bruker Instruments, Billerica, MA). These spectra were acquired at 20° C with a digital resolution of 0.143 Hz/point and a 33° pulse angle. ^{1}H NMR spectra for the 5 and 50mM solutions at a pH of 7.9 and the 50mM solution at a pH of 10.25 are given in Figures 4-8, 4-9 and 4-10, respectively.

^{13}C NMR Studies. A 50mM solution of NaDC was prepared in H_2O/D_2O at a pH of 7.9. A drop of dioxane was added to a portion of the solution and a ^{13}C NMR spectrum was acquired. The spectrum is given in Figure 4-11. After 14 days a drop of dioxane was added to a second aliquot of the solution and a ^{13}C NMR spectrum was acquired. The spectrum is given in Figure 4-12. Dioxane was added to provide an internal reference for chemical shift assignments in the magnetic resonance spectra. The spectra were decoupled with composite pulse decoupling. These spectra were acquired at 20° C with a digital resolution of 0.663 Hz/point, a 33° pulse angle and line broadening of 5 Hz.

4.2.6. Chiral Separation of ±Bi-2-naphthol.

The capillary and buffer vials were filled with a solution of 50mM NaDC at a pH of 8.0. The current was recorded at applied voltages of 0.00, 3.00, 7.00, 10.0, 15.0, 20.0, 22.5, 25.0, 27.5 and 30.0 kV. The Ohm's Law plot of current versus voltage is given in Figure 4-13.

The effect of pH and buffer content on the enantiomeric resolution of ±bi-2-naphthol was investigated. 50mM solutions of NaDC were prepared in 20 mM phosphate buffers at

pH values of 7.50, 7.75, 8.00, 8.25 and 8.50. 50mM solutions of NaDC were prepared in solutions containing 10 mM phosphate and 10 mm borate at pH values of 7.13, 7.61, 7.93, 8.49 and 9.00. 50mM solutions of NaDC were prepared without added buffer at pH values of 7.45, 7.69, 8.15, 8.52 and 9.20. These fifteen solutions were evaluated as a micellar mobile phases in MECC for the chiral separation of ±bi-2-naphthol. A bare fused silica column was used with a 50μm ID, a 350μm OD, and a length of 56 cm to detection and 65 cm overall. The applied voltage was 10 kv. A solution containing racemic bi-2-naphthol was prepared in methanol at a concentration of 1 mg/mL. Hydrodynamic injections were performed for 3 s at a height of 10 cm. Plots of enantiomeric resolution versus pH are given in Figure 4-14 for the three different buffer systems. The electropherogram obtained using a solution of 50mM NaDC at a pH of 8.15, with no added buffer, is given in Figure 4-15.

The effect of NaDC concentration on the enantiomeric resolution of ±bi-2-naphthol was investigated. A 200 mM stock solution of NaDC was prepared at a pH of 8.0. Solutions of NaDC at concentrations of 10, 25, 50, 75, 100 and 150 mM were prepared from the stock solution. These six solutions were evaluated as micellar mobile phases in MECC for the chiral separation of ±bi-2-naphthol using the same column, voltage, sample and injection technique as the previous study. A plot of enantiomeric resolution versus NaDC concentration is given in Figure 4-16.

A stock solution of 50mM NaDC was prepared at a pH of 8.0. An aliquot of this solution was evaluated as a micellar mobile phase in MECC for the chiral separation of ±bi-2-naphthol using the same column, and injection technique as the previous two studies. Applied voltage for all experiments in this study was held at 20 kV. Aliquots of this solution were

evaluated by the same method at times of 1, 2, 4, 5 and 14 days since preparation of the solution. The sample, racemic bi-2-naphthol, was prepared at a concentration of 1 mg/mL in a saturated solution of Sudan III in methanol. A plot of retention times versus solution age for each enantiomer and the Sudan III is given in Figure 4-17. Electropherograms showing the separations observed for the enantiomers of bi-2-naphthol using the freshly prepared solution and after the solution had aged for 14 days are given in Figures 4-18 and 4-19, respectively.

4.3. Results and Discussion

The initial investigations of the chiral separation of dl-laudanosine involved the determination of the effects of pH and ionic strength. A pH of 4.2 was found to be too acidic for the preparation of micellar solutions of NaTDC for MECC. Highly viscous solutions resulted, at all ionic strengths investigated, which could not be used with such small ID capillaries. The effect which pH and ionic strength of the bile salt solutions have on the chiral resolution of dl-laudanosine is observed in Figures 4-3, 4-4 and 4-5. A C_8-Bonded Phase capillary was chosen for these analyses to reduce electroosmotic flow and the interaction of the bile salt and analytes with the capillary wall. Increasing the ionic strength of the buffer from 0.01 to 0.05 resulted in an increase in the resolution of the enantiomers using solutions with a pH of 6.7. A decrease in the resolution was observed when the ionic strength was increased to 0.15. The highest resolution for solutions having a pH of 6.7 was observed using a buffer ionic strength of 0.05. Increasing the buffer ionic strength for a solutions having a pH of 7.9 decreased the resolution of these enantiomers. The best resolution was observed at a pH of 7.9 and a buffer ionic strength of 0.01.

Increases in viscosity of bile salt solutions were visually observed with changes in pH and with increased age of the micellar solutions. These observations led to several studies designed to quantify the changes in viscosity and attempt to correlate these changes to chiral resolution. The results of viscosity measurements of 50mM solutions of the unconjugated bile salt, NaDC, for solutions at various pH values, and over time are given in Figures 4-6 and 4-7, respectively. An steady increase in viscosity was observed for solutions below a pH of 7.75. No significant increase in viscosity was observed with time, up to 14 days for the 50mM NaDC solution.

NMR spectroscopy was reported as a diagnostic tool for NaDC solutions. Changes in linewidths and intensities in the proton and carbon-13 NMR spectra had been reported to be indicators of changes in aggregate properties such as aggregation number and the type of micelle formed (5,6). ^{1}H Spectra were acquired for 5 and 50mM solutions at a pH of 7.9 and a 50mM solution at a pH of 10.25, the spectra obtained are given in Figures 4-8, 4-9 and 4-10, respectively. These spectra contained spectroscopic evidence which supported micelle formation from 5 mM to 50 mM and directly correlated to literature values for NaDC in aqueous solutions (5,6,15). The bile salt molecules in the micellar solutions at 50 mM are expected to have less translational and rotational movement. The relaxation time of the resonance decreases and the peak width observed in the magnetic resonance spectrum broadens. There were no significant differences in chemical shifts, line intensities or bandwidths between the spectra obtained at pH values of 7.9 and 10.25. This indicated that NaDC solutions at pH values of 7.9 and 10.25 have similar aggregation properties. ^{13}C spectra were acquired for a fresh 50mM solution at a pH of 7.9, and the same solution after

it had aged for 14 days. The spectra are given in Figures 4-11 and 4-12. As can be seen from these figures, there were no significant changes in the spectrum after the solution had aged for 14 days. These results indicated that there were no significant changes in the aggregation properties of 50mM NaDC solutions after 14 days.

The upper limit of voltage was established prior to collecting electrophoretic data for the studies involving NaDC solutions . The Ohm's Law plot for a 50mM NaDC solution at a pH of 8.0 is given in Figure 4-13. Positive deviation of the current was observed above 20 kV. The upper limit of voltage was therefore established at 20 kV for capillary electrophoresis experiments using this instrumental setup with 50 mM NaDC solutions. Operating the CE instrument at or below the upper limit of voltage reduces the likelihood of joule heating of the solution in the capillary tube as discussed in Chapter 1.

The separation of the enantiomers of bi-2-naphthol was used to evaluate solution variables when using NaDC as a chiral selector in MECC. Figure 4-14 is a plot of resolution versus pH for 50 mM NaDC solutions prepared without buffer and with two different buffers. The highest values of chiral resolution for solutions containing a phosphate buffer or a phosphate/borate buffer were observed for solutions at the lowest values of pH tested. Figure 4-16 is a plot of resolution versus bile salt concentration. It shows the effect of NaDC concentration on the chiral resolution of bi-2-naphthol. The highest chiral resolution was observed at a concentration of 50mM. The highest overall chiral resolution was observed using a solution without added buffer at a pH of 8.15. Figure 4-15 is an electropherogram obtained using this solution. A clear baseline separation with a resolution value of 2.1 is observed.

A study of the effect of micellar solution age on the chiral resolution of bi-2-naphthol was performed using a 50mM NaDC solution at a pH of 8.0. A plot of retention times versus solution age for the enantiomers of bi-2-naphthol and Sudan III, a micelle time marker, is given in Figure 4-17. The migration times of all three compounds increased over time. A comparison of the electropherograms in Figures 4-18 and 4-19 shows the greatest difference in the retention times. It can also be observed in Figure 4-17 that the variability in the retention times for duplicate analyses also began to increase significantly 14 days after preparation.

4.4. Conclusions

The separation of dl-laudanosine is affected by the pH and ionic strength of the NaTDC micellar solution. The resolution reported by Nishi, Fukuyama, Matsuo and Terabe for the enantiomers of laudanosine is about 0.8 using a bare fused silica capillary and a phosphate/borate buffer (3). The highest resolution observed here was calculated to be 1.1 when using a phosphate buffer and a coated capillary. The experiments performed prove that it is possible to reproduce chiral separations reported in the literature using the laboratory assembled instrument.

Changes in the NaDC solutions were observed when the surfactant concentration was increased from 5 to 50mM and when the pH was lowered below 7.75. No significant changes in the solution were observed after 14 days. The separation of ±bi-2-naphthol changed with many of the conditions studied, including the time since solution preparation. The best resolution for the enantiomers of bi-2-naphthol was observed using a solution containing 50mM NaDC with no buffer at a pH of 8.0. At the time this work was completed, the best

chiral resolution for bi-2-naphthol reported in the literature was a value of 1.5 using a 50mM solution of NaDC with 12% methanol. This solution also contained a phosphate and borate buffer at a pH of 9.0. The observed baseline resolution of 2.1 for the enantiomers of bi-2-naphthol was as good if not better than the reports of separations of these enantiomers in the literature using MECC with bile salts. The optimization of pH and the adjustment of pH without added buffer provides an improved environment for the NaDC solution to act as a chiral pseudophase in MECC.

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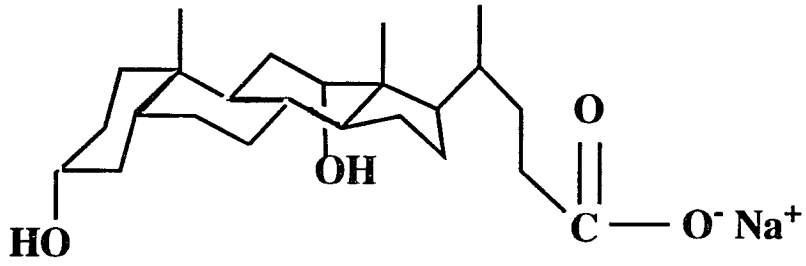
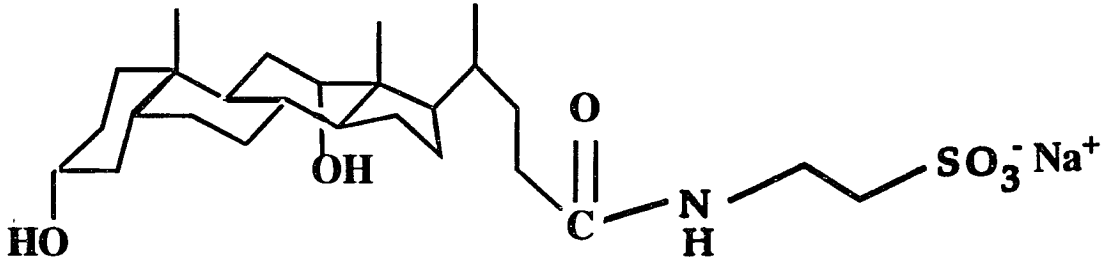
Figure 4-1. Structures of Bile Salts

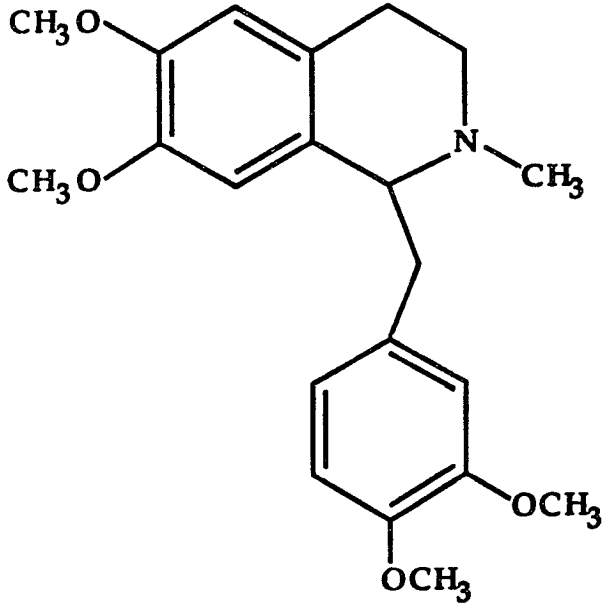


Sodium Deoxycholate

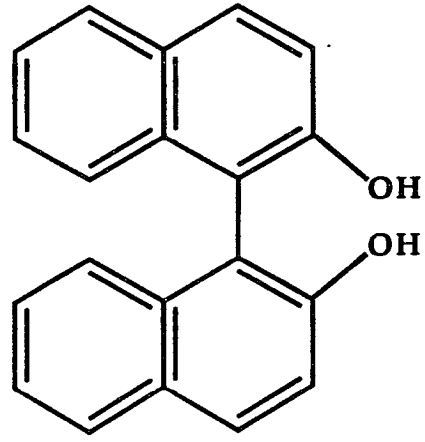


Sodium Taurodeoxycholate

Figure 4-2. Structures of Solutes



DL - Laudanosine



R-(-)-1,1'-Bi-2-naphthol

Figure 4-3. Effect of Ionic Strength on the Chiral Resolution of DL-Laudanosine using 50mM Sodium Taurodeoxycholate at pH = 6.7 and pH = 7.9.

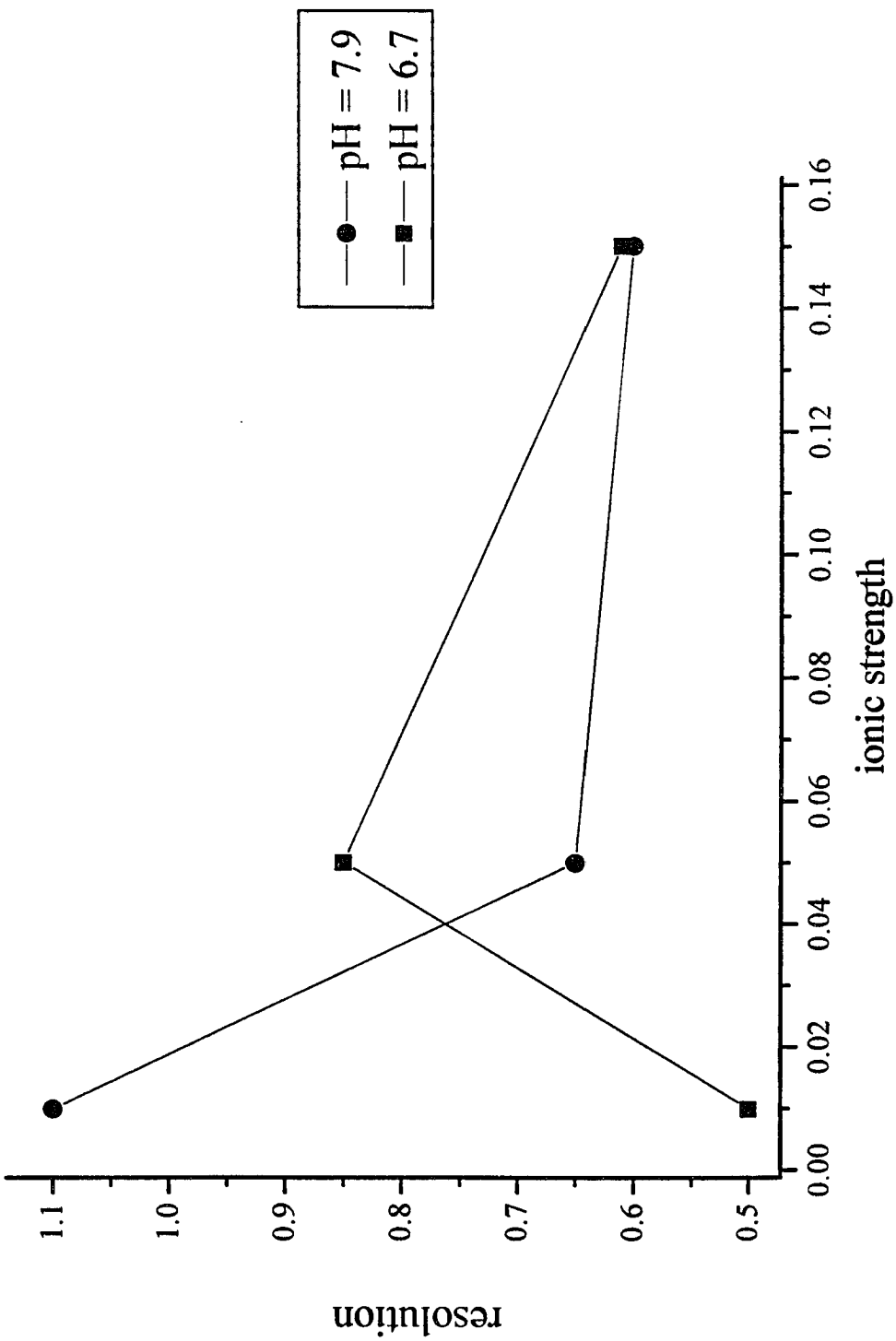


Figure 4-4. Effect of Buffer Ionic Strength on the Chiral Separation of DL-Laudanosine Using Sodium Taurodeoxycholate at a Buffer pH of 6.7

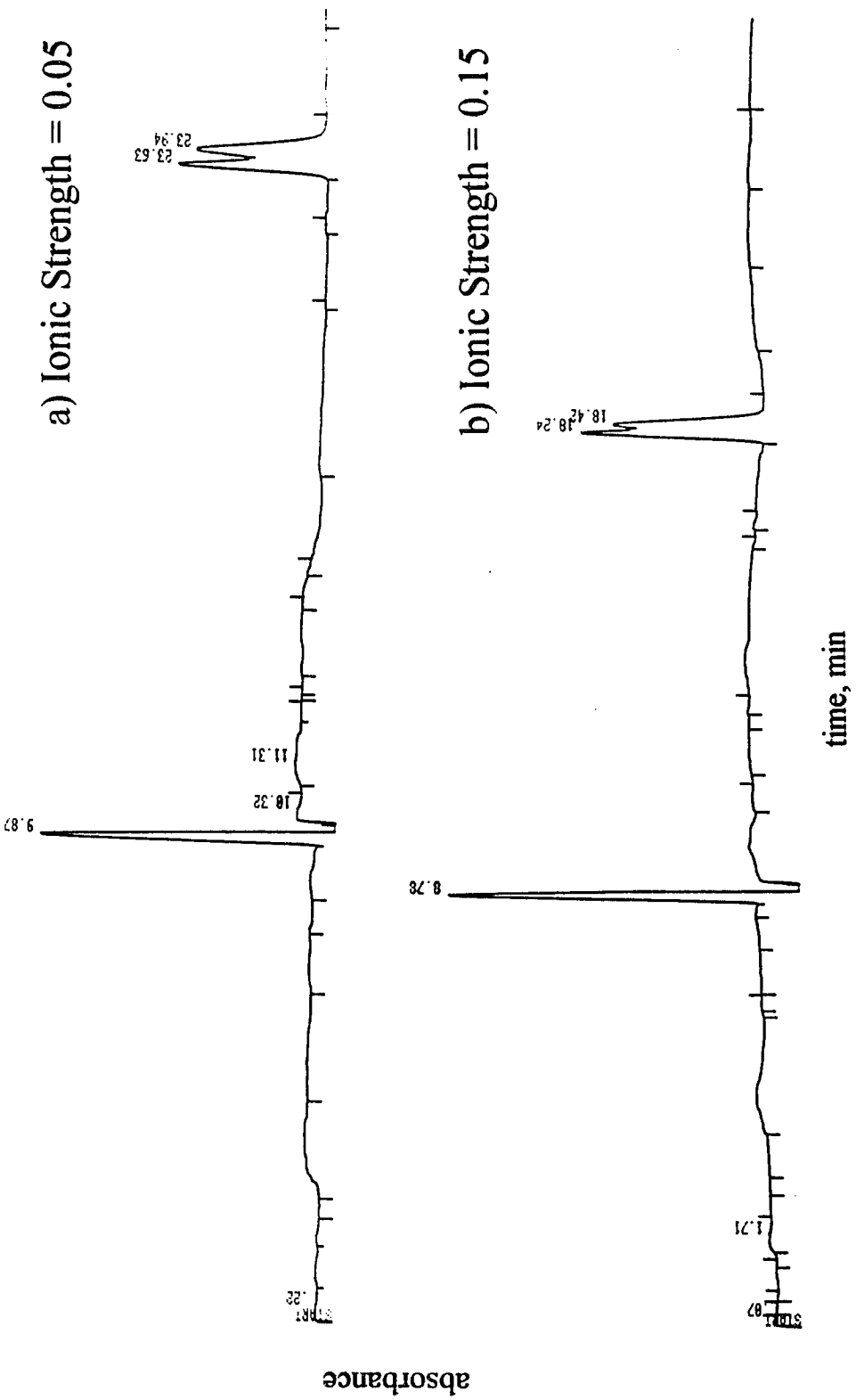
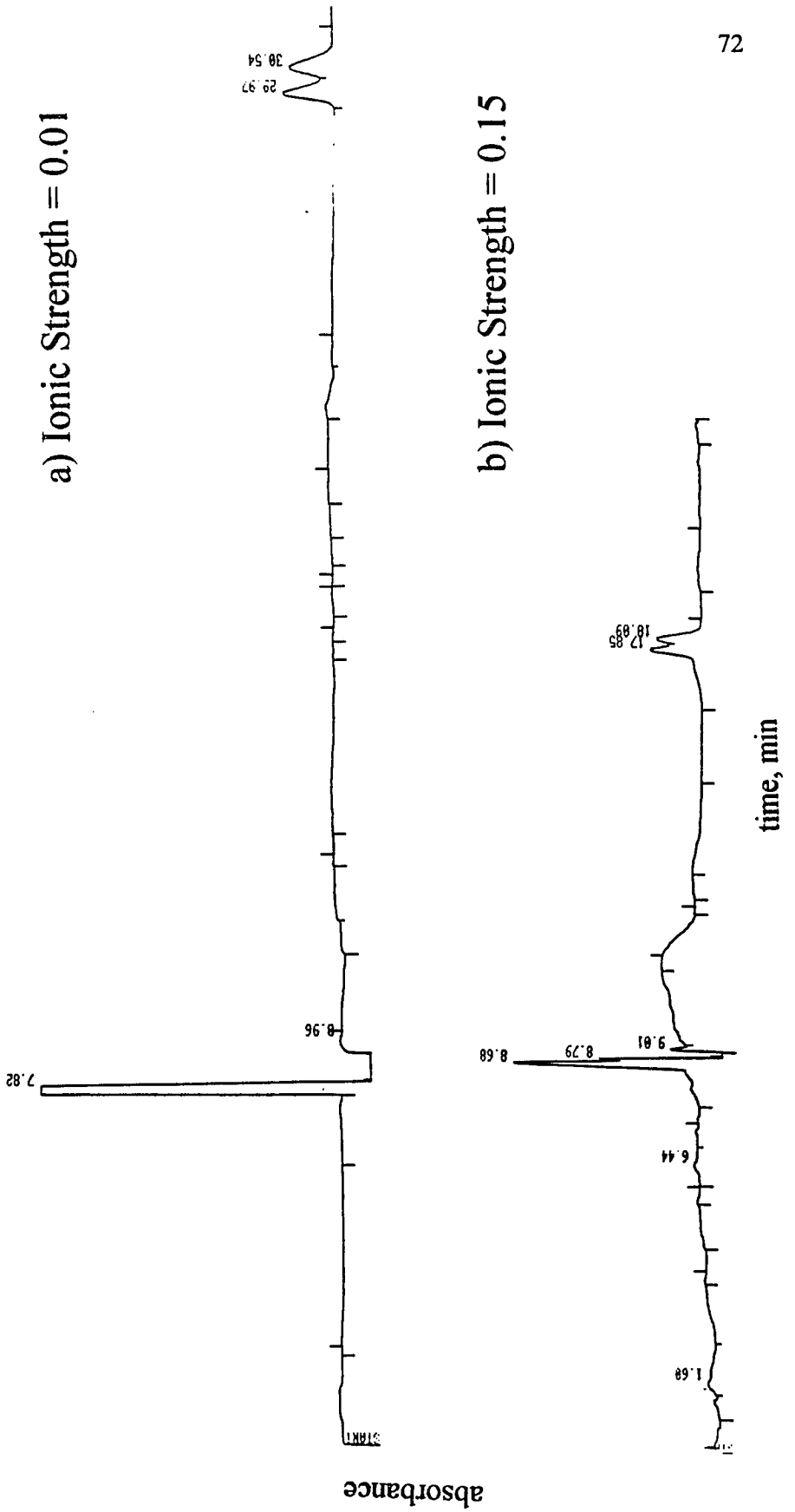


Figure 4-5. Effect of Buffer Ionic Strength on the Chiral Separation of DL-Laudanosine Using Sodium Taurodeoxycholate at a Buffer pH of 7.9



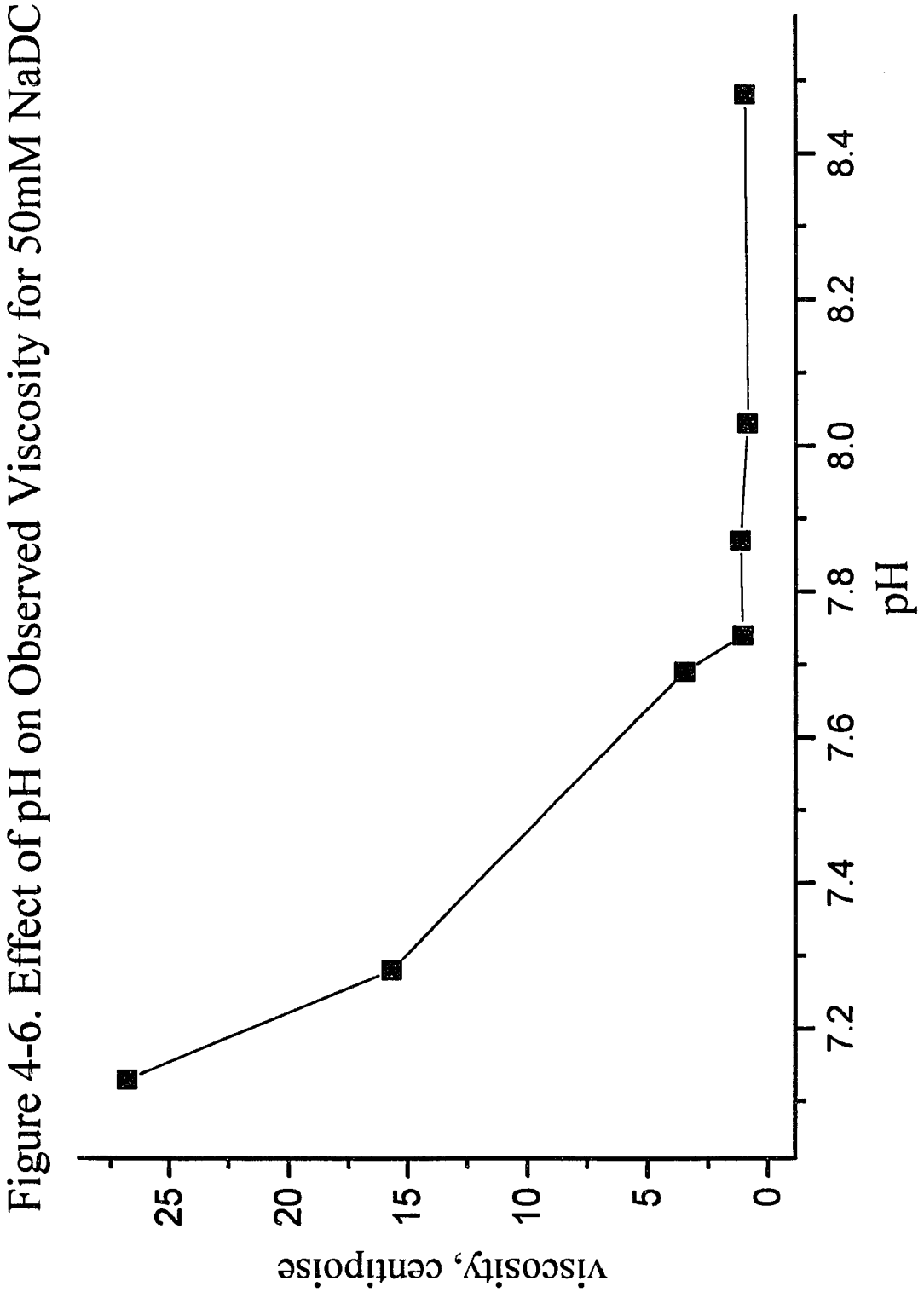


Figure 4-6. Effect of pH on Observed Viscosity for 50mM NaDC

Figure 4-7. Effect of Solution Age on Observed Viscosity for 50mM NaDC

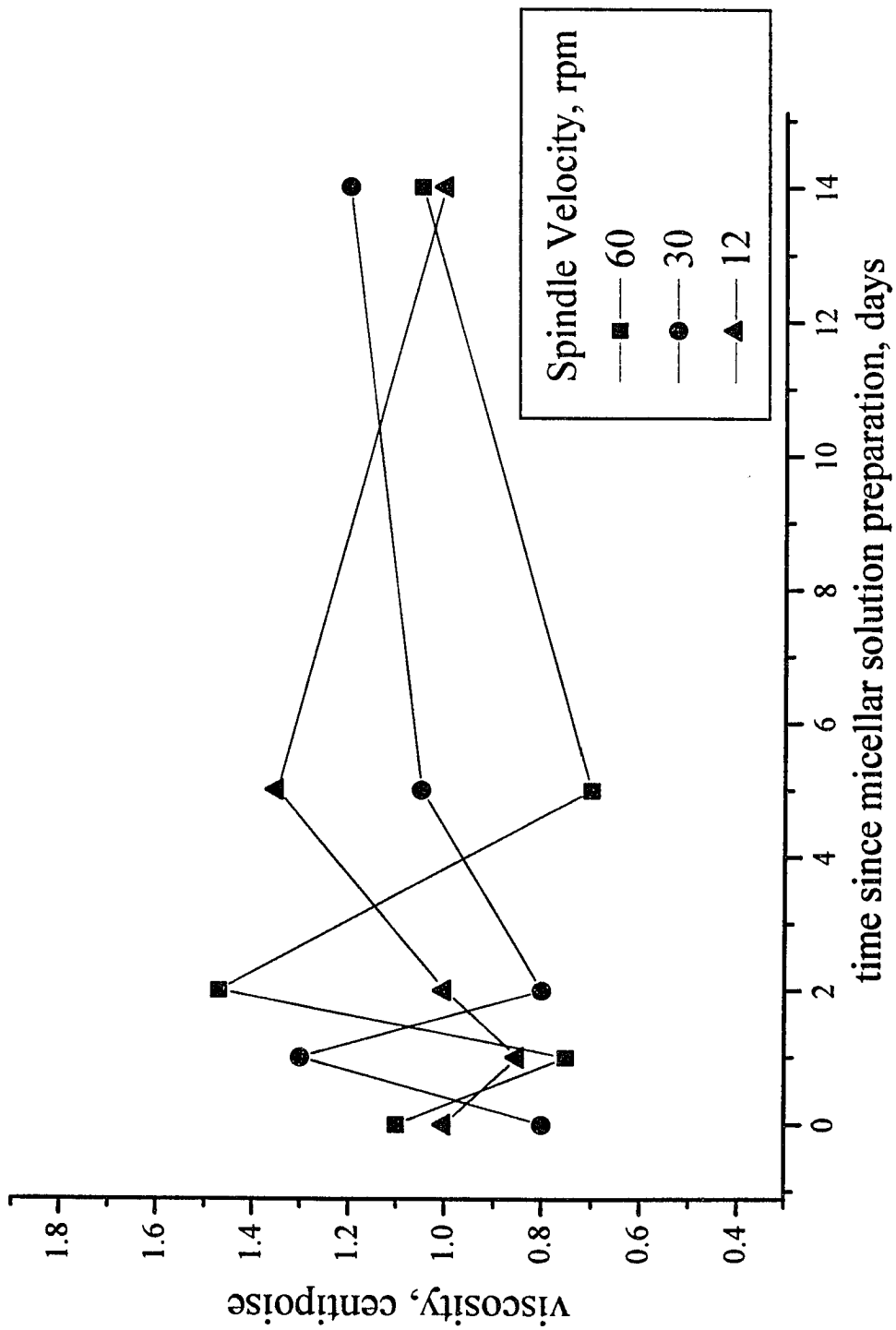


Figure 4-8. ^{1}H NMR Spectrum of 5mM NaDC at a pH of 7.9.

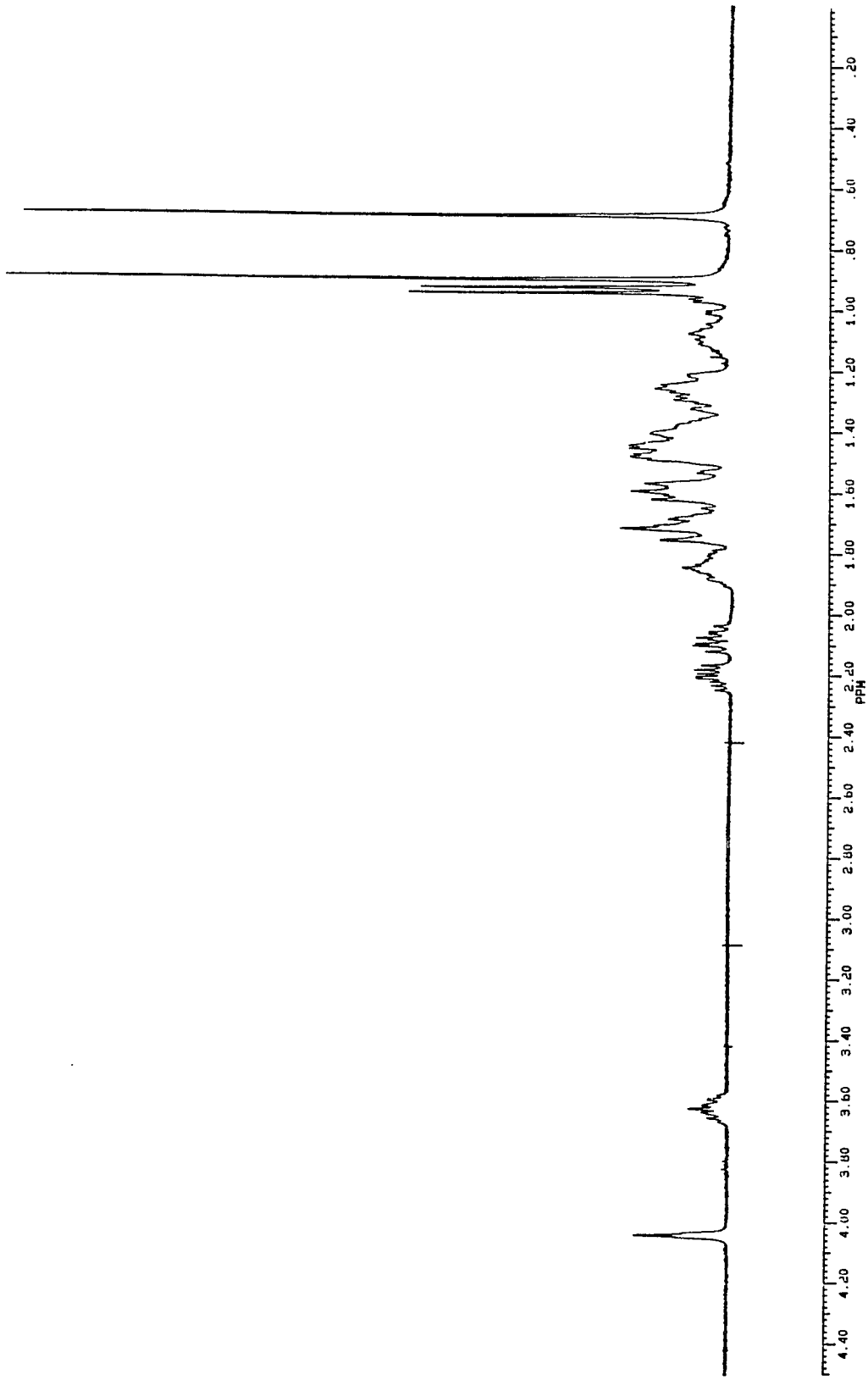


Figure 4-9. ^{1}H NMR Spectrum of 50mM NaDC at a pH of 7.9.

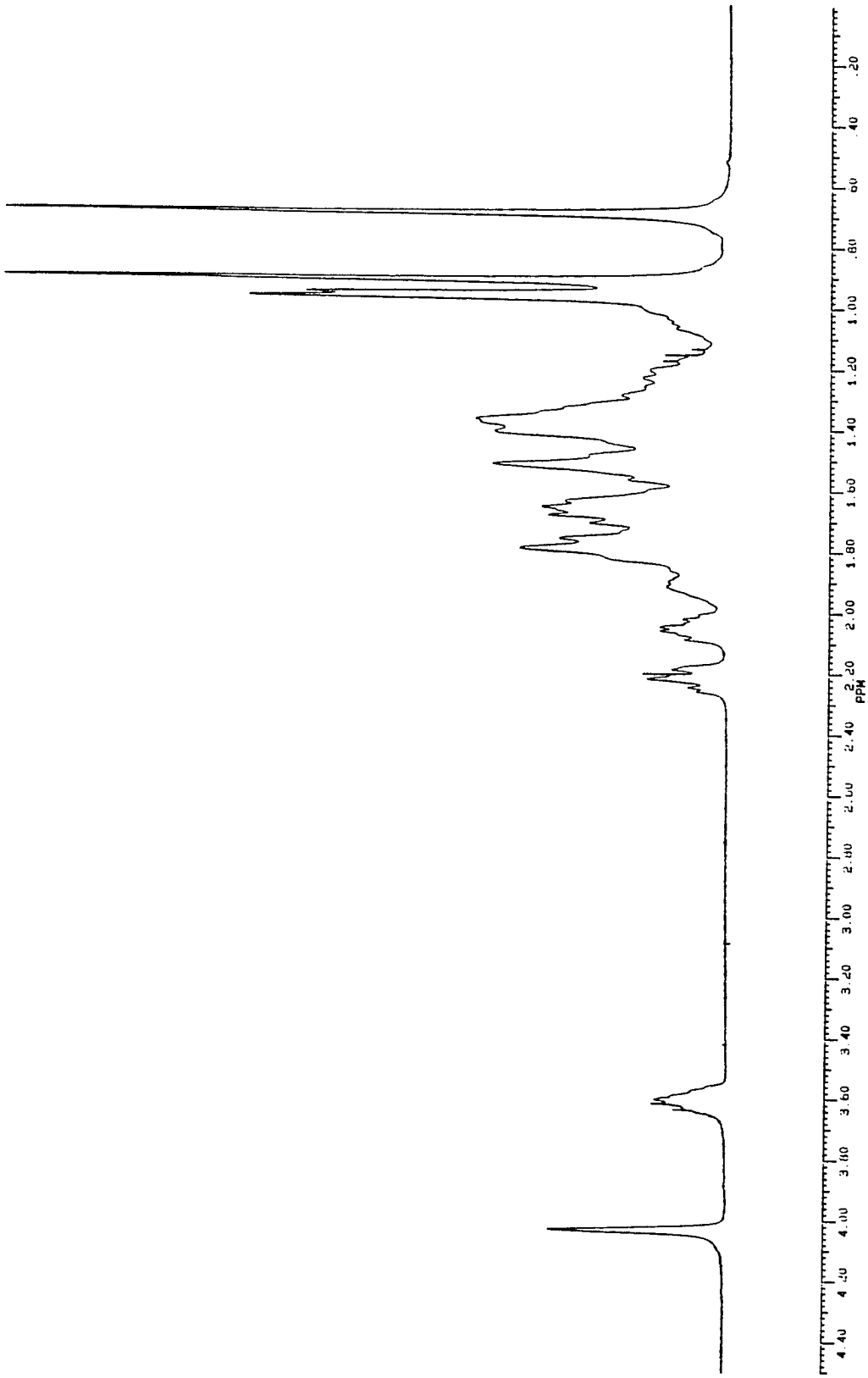


Figure 4-10. ^{1}H NMR Spectrum of 50mM NaDC at a pH of 10.25.

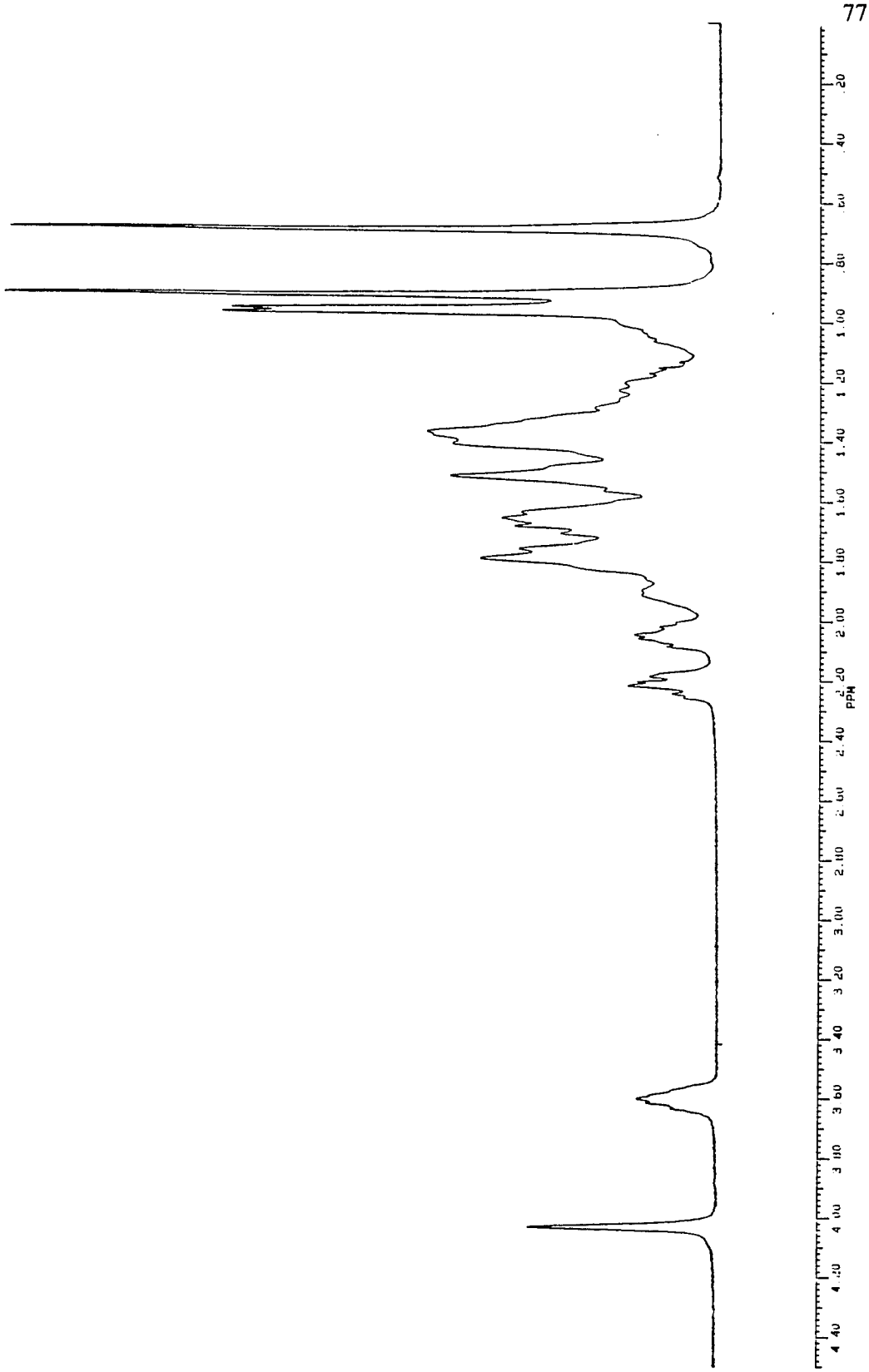


Figure 4-11. ^{13}C NMR Spectrum of 50mM NaDC at a pH of 7.9.

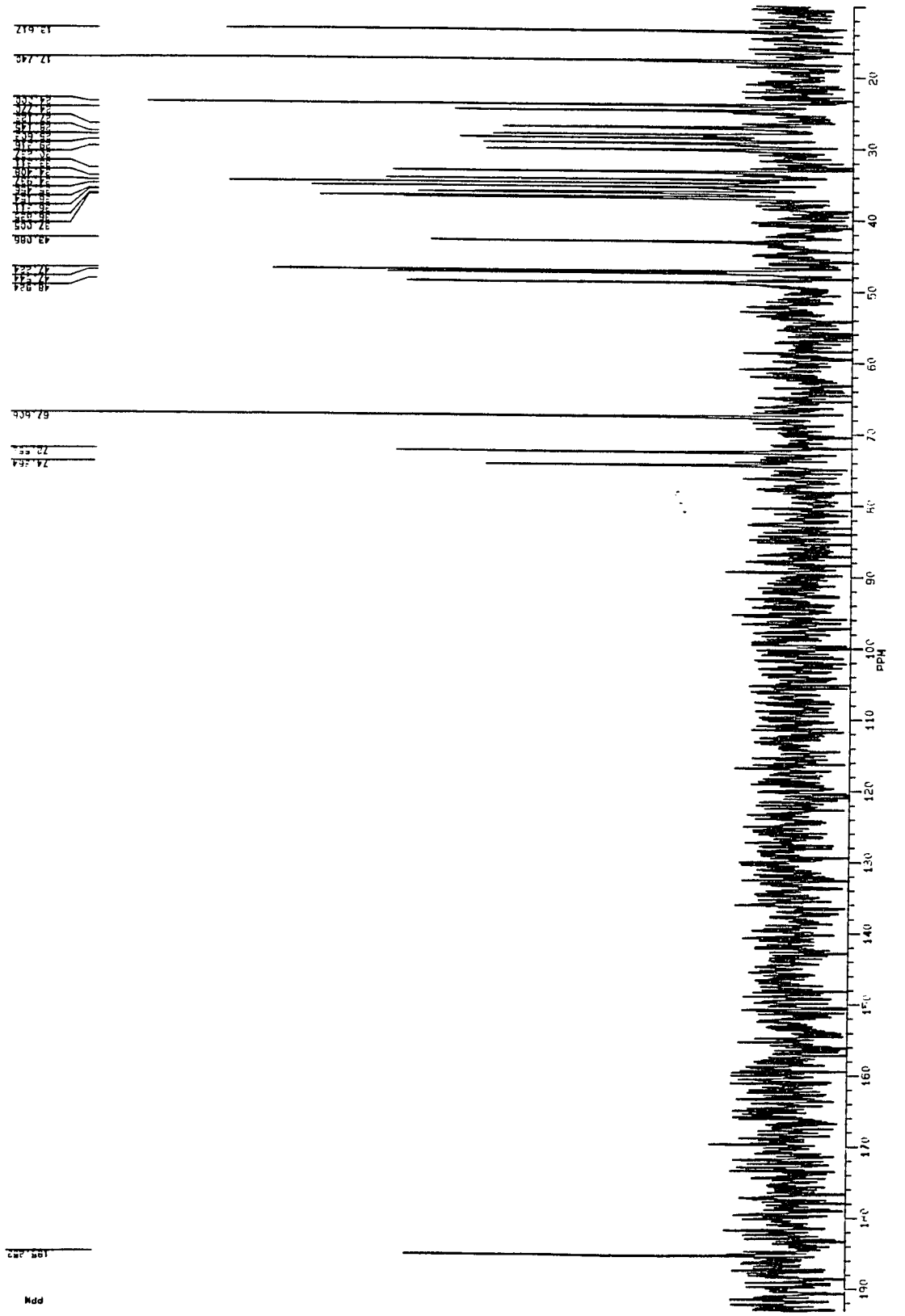
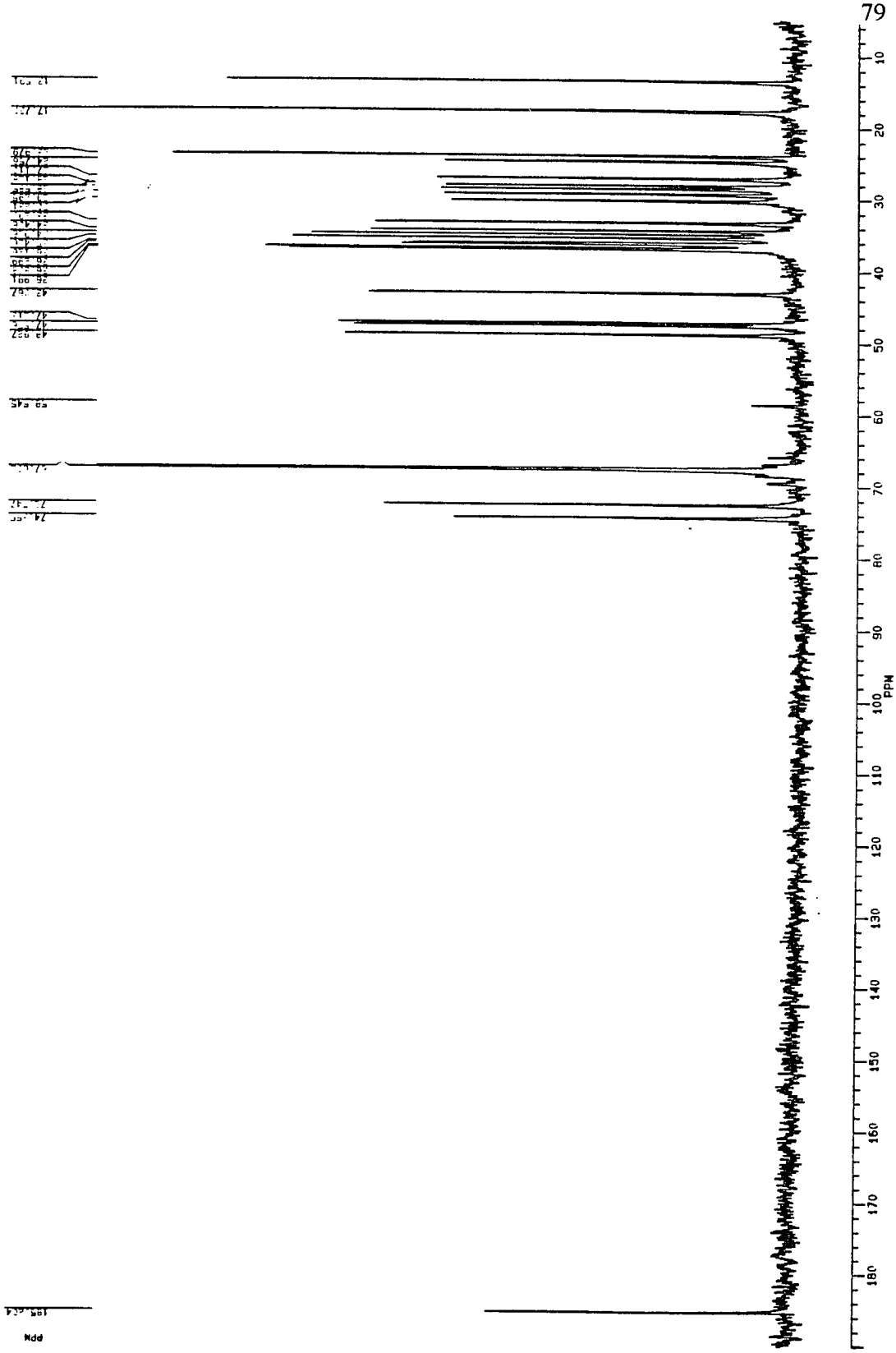


Figure 4-12. ^{13}C NMR Spectrum of 50mM NaDC at a pH of 7.9 After 14 Days.



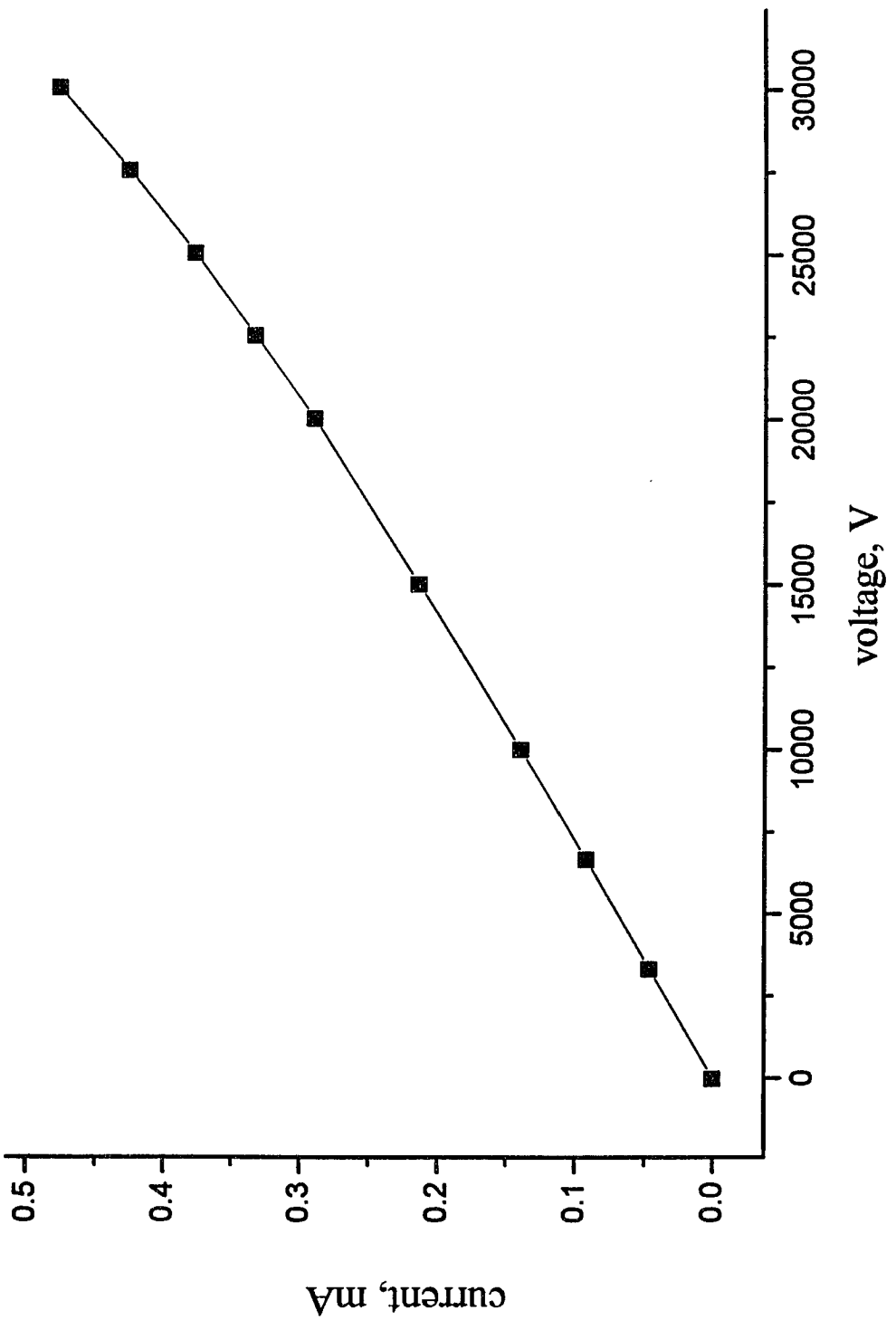
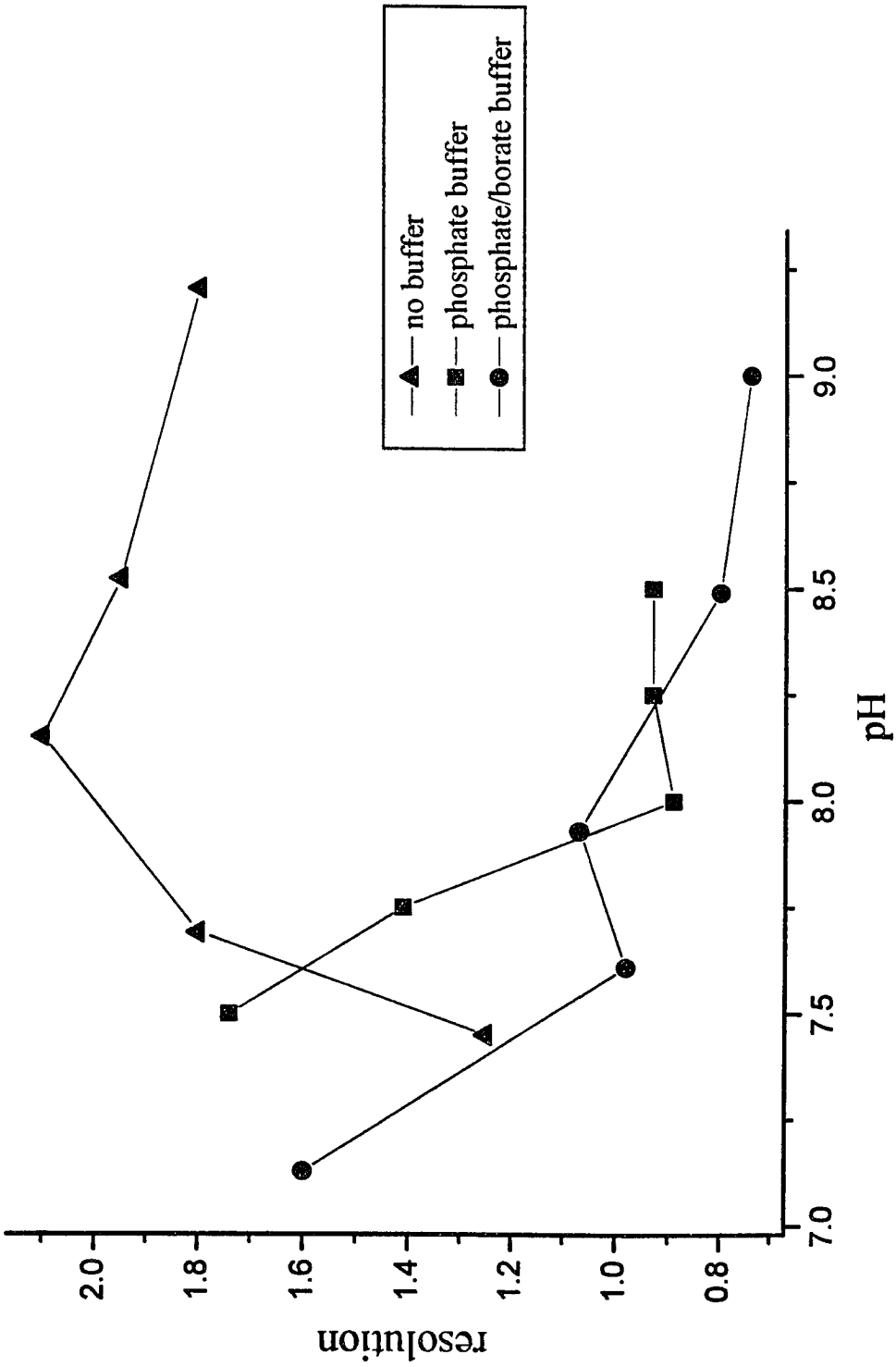


Figure 4-13. Ohm's Law Plot for 50mM NaDC

Figure 4-14. Effect of pH on the Resolution of Bi-2-naphthol Enantiomers in 50mM Sodium Deoxycholate Solutions with and without added buffer.



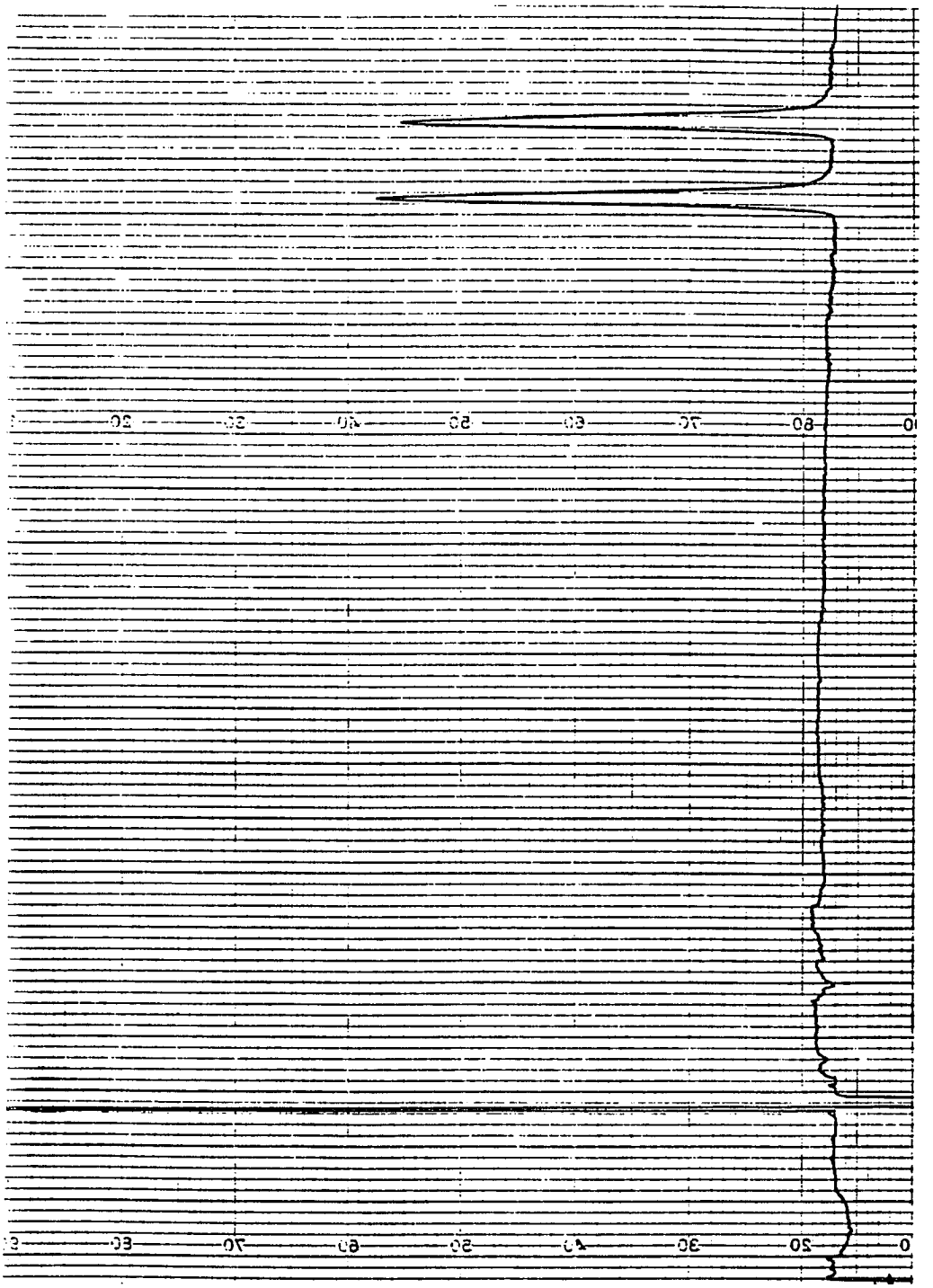


Figure 4-15. Capillary Electropherogram of Racemic Bi-2-naphthol Obtained Using 50mM NaDC at a pH of 8.15.

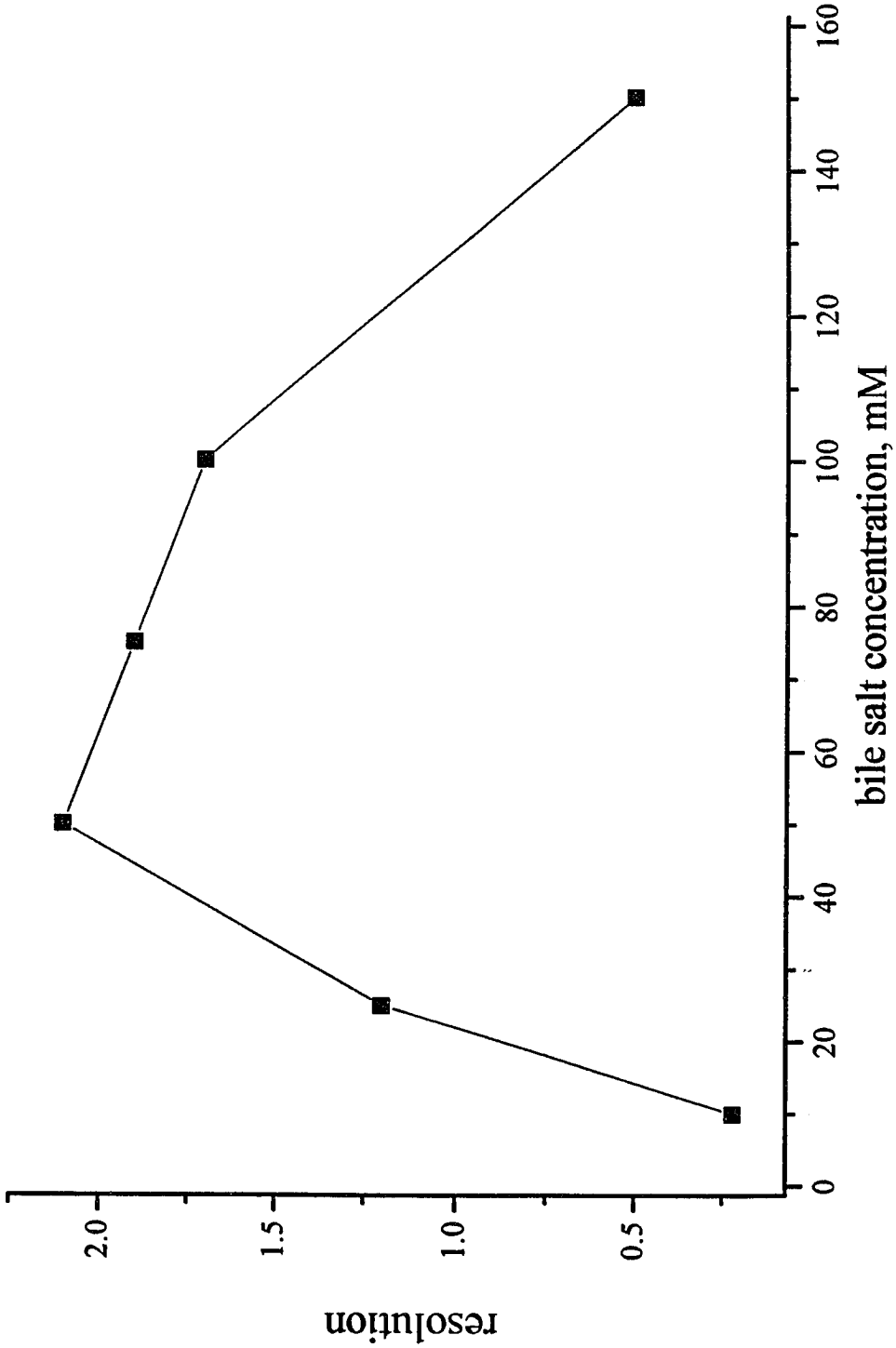


Figure 4-16. Effect of bile salt concentration on the enantiomeric resolution of bi-2-naphthol using solutions containing sodium deoxycholate.

Figure 4-17. Variability in Migration Times versus Micellar Solution Age for 50mM NaDC at pH 8.0, 10kV applied voltage

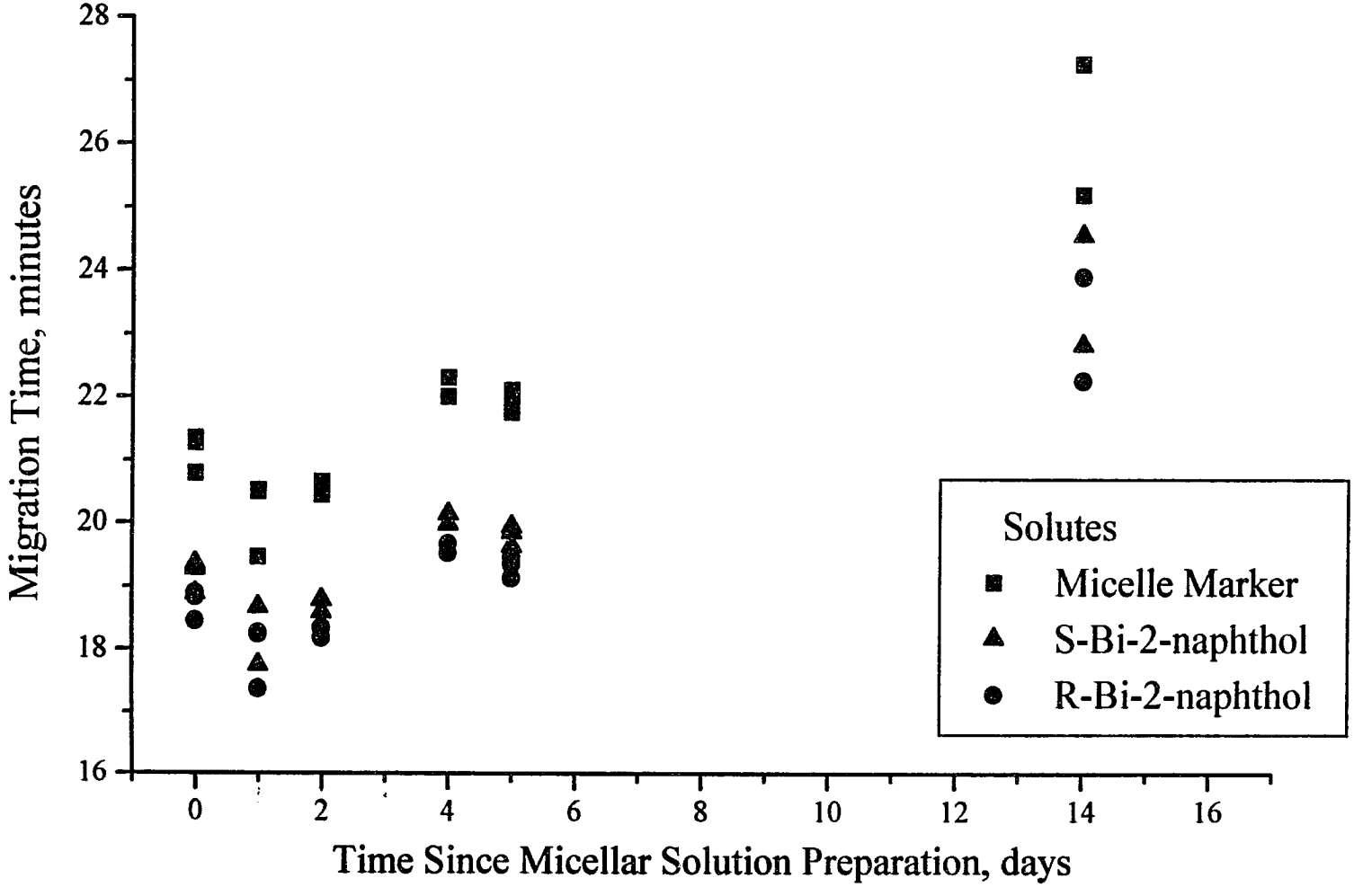


Figure 4-18. Separation of ±Bi-2-naphthol Using a Freshly Prepared 50mM Solution of NaDC, pH = 8.0

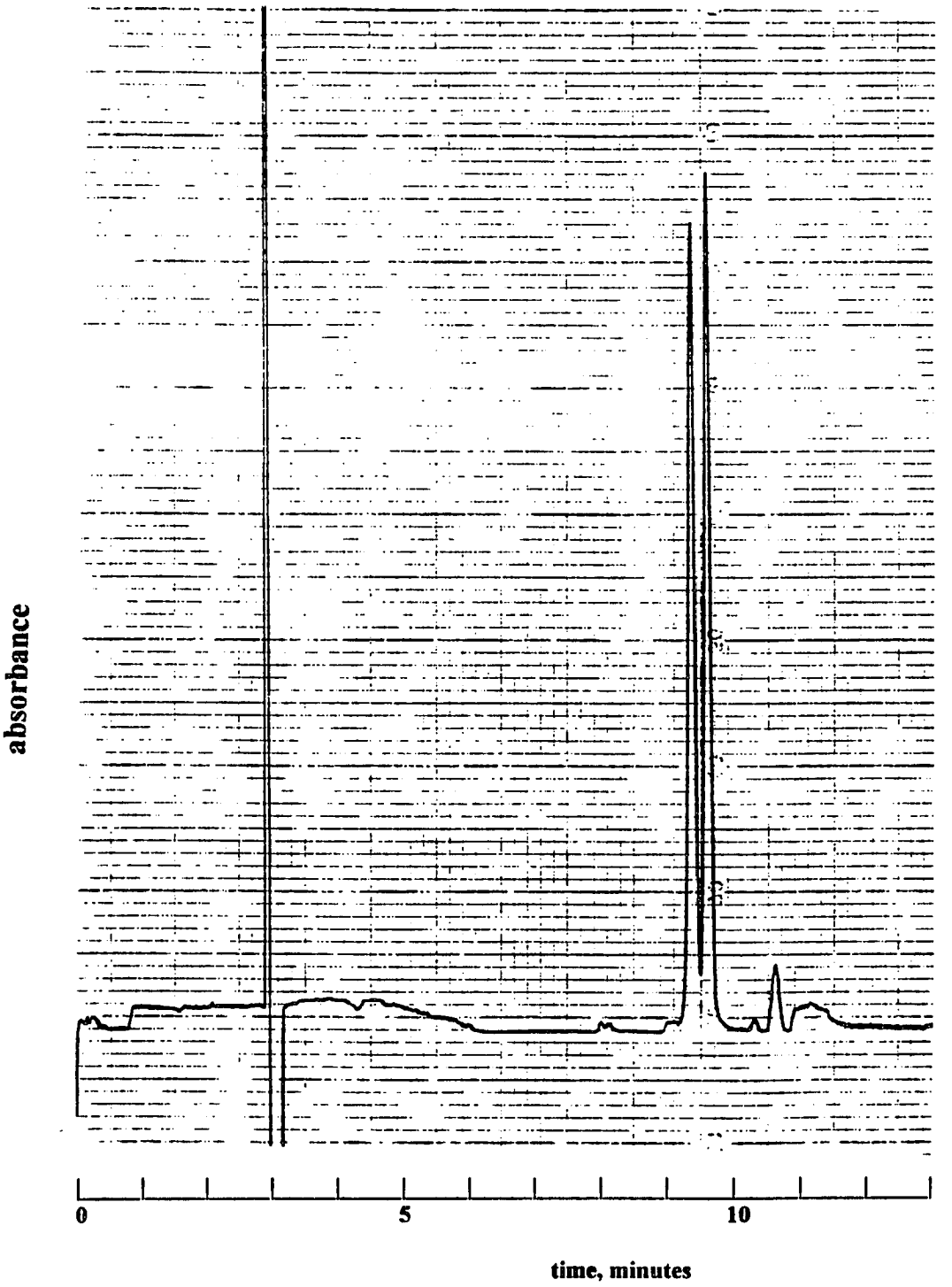
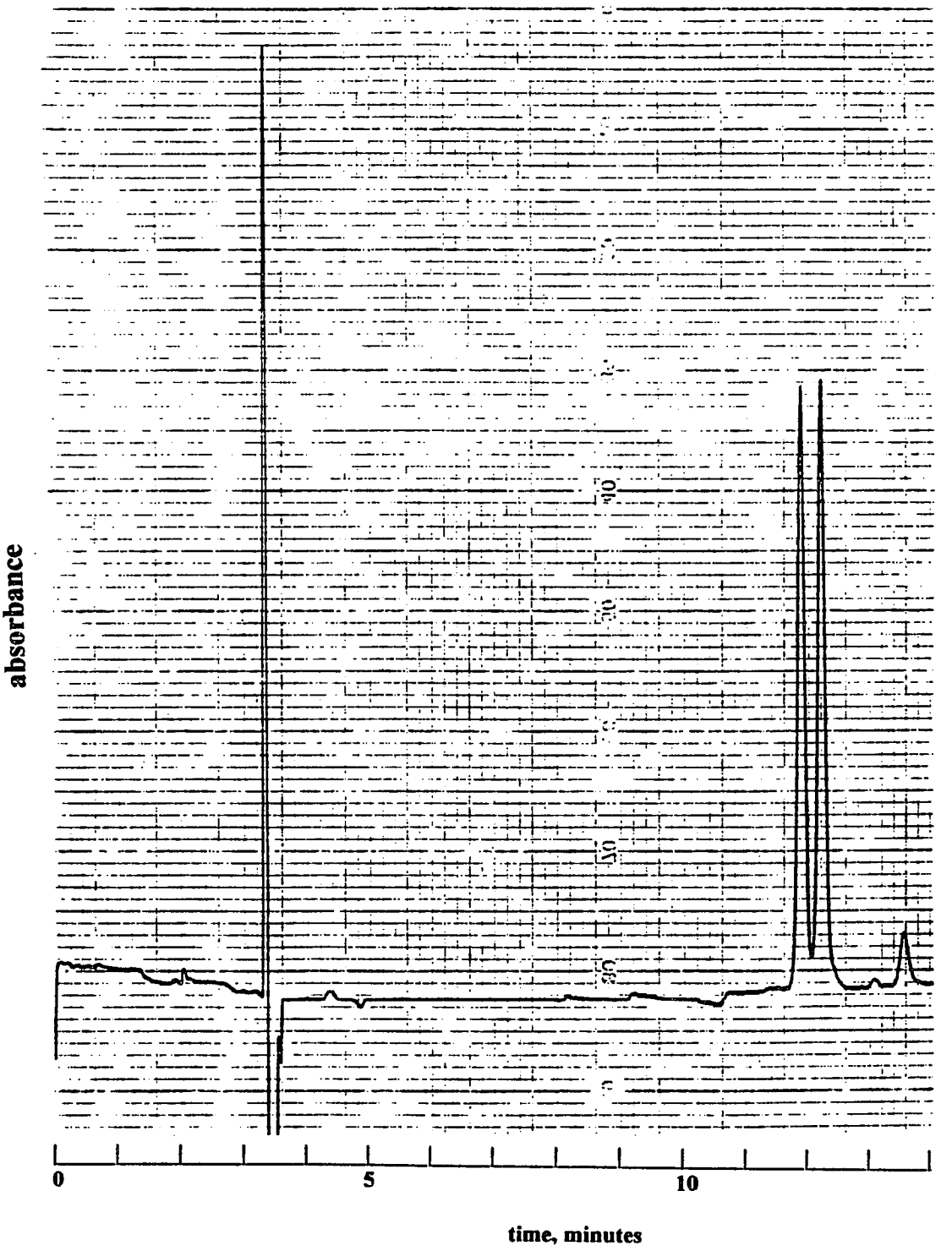


Figure 4-19. Separation of ±Bi-2-naphthol Using a 14 Day Old 50mM Solution of NaDC, pH = 8.0



CHAPTER 5

CHIRAL SEPARATION OF VERAPAMIL AND RELATED COMPOUNDS USING MECC WITH MIXED MICELLES OF BILE SALT AND POLYOXYETHYLENE ETHERS

5.1 Introduction

The bile salts are naturally occurring chiral surfactants which have been used successfully as mobile phase modifiers in micellar electrokinetic capillary chromatography (MECC) for enantiomeric separations (1-8). Mixed micelles of bile salt and sodium dodecyl sulfate have also been used as mobile phase modifiers in MECC (9). The aggregation behavior of the bile salts under various conditions has been studied using light scattering (10,11), nuclear magnetic resonance, electron spin resonance and x-ray techniques (12-15). The literature values for the critical micelle concentrations (CMC) and aggregation numbers (AN) for the bile salts vary widely. A review by Kratohvil indicates that purification of the bile salts is of the utmost importance for obtaining reproducible data on the aggregation of these molecules (16). The author gives the accepted values for the CMC and AN of the unconjugated, dihydroxy bile salt, sodium deoxycholate (NaDC), as 2.4 mM and 7, respectively, in a solution containing 0.149 M NaCl. This aggregation number is still relatively small when compared to many other surfactants used as pseudophases in MECC. Sodium dodecyl sulfate, for example, has an AN of 64 under similar conditions (17).

The bile salts and polyoxyethylene ethers have been shown to form mixed micelles. The properties of these mixed micelles have been investigated (18-22). The addition of polyoxyethylene-8-decyl ether ($C_{10}E_8$) in various mole ratios to NaDC has been shown to alter

the CMC, the AN of the micelles, and the ability of the micelles to solubilize cholesterol. For example, a mixed solution of the ether $C_{10}E_8$ and NaDC containing an ether mole fraction of 0.25, was observed to have a CMC of 1.63 mM and an AN of 25. A solution containing NaDC was observed to have a CMC of 3.16 mM and an AN of 18 under the same experimental conditions (22). Asano and coworkers indicate that the hydrophobicity of the interior of the mixed micelle decreases with increased mole fraction of ether. For example, increasing the mole fraction of ether from 0.00 to 0.43 in mixed solutions of NaDC and $C_{10}E_8$ results in the formation of micelles which decreased in hydrophobicity (22). A steady increase in the solubilization of cholesterol was observed for mixed solutions of NaDC and $C_{10}E_8$ when the mole fraction of ether was increased from 0.00 to 0.40.

It was our goal to determine the ability of mixed micelles of bile salt and polyoxyethylene ethers to act as a pseudophase in MECC for chiral separations. These studies allowed us to gain an understanding of separations using mixed micelles and further expand the applications of bile salt MECC. The compounds chosen for the study were verapamil, norverapamil, gallopamil and bi-2-naphthol (the structures for verapamil and related compounds are given in Figure 5-1). The separation for the enantiomers of bi-2-naphthol using bile salts with methanol as a mobile phase modifier in MECC is well documented (23). The separation of the enantiomers of bi-2-naphthol using mixed micellar solutions and mixed micellar solutions with methanol provided additional reference points for comparison to solutions of bile salt and bile salt with methanol.

Enantiomeric separation of the widely administered calcium ion channel blocking drug, verapamil, its major metabolite, norverapamil, and a methoxy derivative, gallopamil, has

been an area of active research (24-30). Chiral resolution of the verapamil enantiomers has been achieved with HPLC using several chiral stationary phases, including α_1-acid glycoprotein (24,25), amylose tris-3,5-dimethylphenylcarbamate (25), cyclodextrin (26), ovomucoid (27), and cellulose based (28, 29) columns. Chiral resolution for verapamil has been demonstrated with MECC using cyclodextrins with anionic surfactants (30). Enantiomeric resolution has also been observed using some of the chiral stationary phases for norverapamil (24-26) and gallopamil (24). The simultaneous separation and enantiomeric determination of verapamil and norverapamil have been reported using two separation schemes. The first involves coupled column achiral-chiral HPLC (24, 26). The second method involves derivatization of the norverapamil followed by chiral HPLC (25).

Experiments have been performed to determine the conditions under which mixed micelles of NaDC and each of the three ethers $C_{12}E_4$, $C_{10}E_8$, and polyoxyethylene-6-dodecyl ether ($C_{12}E_6$) enhance the chiral separations of verapamil and bi-2-naphthol. The results of these investigations have provided the conditions which allow simultaneous baseline separation of the enantiomers of verapamil, norverapamil, and gallopamil using MECC.

5.2 Experimental

5.2.1 Apparatus for CE

Studies were conducted using both a laboratory assembled capillary electrophoresis instrument and a HPCE-3D (Hewlett-Packard, Wilmington, DE) system. The laboratory assembled instrument was described in Chapter 2. Experiments were performed using 50 micron ID bare fused silica capillaries (Supelco, Bellefonte, PA). The absorbance at 210nm was recorded using both a strip chart recorder (Kipp & Zonen, Holland) and a model 3390A

integrator (Hewlett-Packard). Additional experiments for verapamil and bi-2-naphthol were performed using the HPCE-3D system with a HP bare fused silica capillary to confirm the results obtained with the laboratory assembled instrument.

5.2.2 Materials

Sodium deoxycholate (NaDC) was purchased from Aldrich Chemical Co. (Milwaukee, WI). The manufacturer-stated purity of NaDC was greater than 98%. The bile salt was recrystallized from ethanol prior to use (16). Polyoxyethylene ethers and bi-2-naphthol enantiomers were purchased from Aldrich and used as received. The R and S enantiomers of verapamil hydrochloride and methoxyverapamil hydrochloride (gallopamil hydrochloride) were purchased from Research Biochemicals Inc. (Natick, MA.). The R and S enantiomers of norverapamil were kindly provided by Dr. L. Miller (Searle Chemical Sciences Department, Skokie, IL). Reagent grade sodium hydroxide, sodium chloride and HPLC grade methanol were purchased from Fisher Scientific (Pittsburgh, PA). Sudan III, a widely used micelle marker, was purchased from Central Scientific (New York, NY). Deionized, distilled water was used for the preparation of all solutions.

5.2.3 Experimental Technique

Prior to performing each electrophoretic run, the capillary was rinsed with a 0.1 M sodium hydroxide solution for two minutes. The surfactant solution used for the experiment was rinsed through the capillary for an additional 2 minutes by applying pressure to a vial containing the solution on the cathode side of the capillary. Injection of analytes was performed hydrodynamically when using the laboratory constructed CE instrument. The injection was performed by raising the cathode end of the column to a height of 15 cm above

the anode for a specified time interval from 1 to 6 seconds. Injections, when using the HPCE system, were performed hydrodynamically by applying pressure to the vial containing the analyte solution.

The capillary used for experiments with the laboratory constructed instrument had an overall length of 75 cm with a length of 65 cm to detection. The capillary used for experiments with the HPCE instrument had an extended light path. The capillary internal diameter of 50 microns was increased to 100 microns for the detection window. The capillary used had an overall length of 64.5 cm with a length of 56 cm to detection. The applied voltage was held constant at 20 kV for both instruments. Typical experimental runs involved rinsing the column with the NaOH solution, followed by the surfactant solution, injecting the solution containing the solute(s) of interest, then simultaneously switching the voltage on and starting the data collection.

The resolution reported is based on baseline width measurements from the electropherograms. This facilitated comparison of the data collected both on the laboratory assembled CE and the HPCE instruments. The HPCE software contained resolution calculation algorithms which resulted in higher resolution values than the manual calculation results reported here.

5.2.4 Solution Preparation

All experiments were performed using solutions with a total surfactant concentration of 50mM. Surfactant solutions were prepared by mixing appropriate volumes of stock solutions containing each type of surfactant. The solutions containing methanol were prepared in the indicated percentages by volume. Polyoxyethylene ether stock solutions were prepared

by dissolving the appropriate quantity of the surfactant in an aqueous solution containing 16 mM NaCl to achieve a final surfactant concentration of 50 mM. NaCl was added to provide a near constant current in all capillary electrophoresis experiments. A 100 mM NaDC stock solution was prepared by dissolving the appropriate quantity of the bile salt in water. Mixed micellar solutions were prepared by combining appropriate volumes of the NaDC stock solution, the polyoxyethylene ether stock solution, methanol and water. The pH of the resultant solutions was between 8.1 and 8.3. Solutions of verapamil, norverapamil, gallopamil and bi-2-naphthol in methanol were prepared from the individual enantiomers. The injected concentration of each enantiomer was 0.25 mg/mL. A fat soluble dye, Sudan III, was added to provide an indication of the micellar migration time.

5.2.5 Experimental Conditions Investigated

The mole fractions of ether and volume percentages of methanol for the solutions studied are given in Table 5-1.

5.3 Results

MECC studies were conducted using solutions containing NaDC alone, in binary mixtures with ether or methanol, and in ternary mixtures with ether and methanol. The studies included solvent solutions having 5, 15 and 25 percent methanol by volume at four ether mole fractions as detailed in Table 5-1. Plots of the resolution obtained for the enantiomers of verapamil versus percent methanol for each mole fraction of $C_{12}E_4$, $C_{12}E_6$, and $C_{10}E_8$ are given in Figures 5-2, 5-3 and 5-4, respectively. Plots of the resolution obtained for the enantiomers of bi-2-naphthol versus percent methanol for each mole fraction of $C_{12}E_4$, $C_{12}E_6$, and $C_{10}E_8$ are given in Figures 5-5, 5-6 and 5-7, respectively. An electropherogram

obtained using the laboratory assembled instrument for the separation of the enantiomers of verapamil, norverapamil and gallopamil under mixed micellar conditions is given in Figure 5-8. Electropherograms obtained using the HPCE instrument for the separation of the enantiomers of verapamil and bi-2-naphthol under mixed micellar and bile salt micellar conditions are given in Figures 5-9 and 5-10, respectively. The number of theoretical plates calculated for (-)-verapamil were 280,000 and 440,000, from the electropherograms in Figures 5-9 and 5-10, respectively.

5.4 Discussion

5.4.1 Evaluation of Mobile Phases Containing $C_{12}E_4$

Mixed surfactant solutions containing $C_{12}E_4$ and NaDC were shown to increase resolution for the verapamil enantiomers and decrease resolution for the bi-2-naphthol enantiomers compared to solutions containing NaDC alone. The structures of verapamil and bi-2-naphthol are quite different. The enantiomers of bi-2-naphthol do not have an asymmetric carbon. Verapamil has an asymmetric carbon which distinguishes the R and S enantiomers. As shown in the plots given in Figure 5-2, enantiomeric resolution was not observed for verapamil using a solution containing only NaDC at a concentration of 50 mM. As shown in the plots given in Figure 5-5, baseline resolution was achieved for the enantiomers of bi-2-naphthol using the same solution. Several reports in the literature indicate larger and less hydrophobic mixed micelles are formed in solutions containing both polyoxyethylene ether and bile salt (18-22). Four plots are given in Figure 5-2 showing the observed enantiomeric resolution of verapamil versus methanol percentage for solutions of NaDC and three mixed micellar ratios with $C_{12}E_4$. The three levels investigated were mole fractions of 0.2, 0.3 and

0.4 $C_{12}E_4$. These data show an increase in resolution for the verapamil enantiomers using the mixed micelles. As the mole fraction of $C_{12}E_4$ increased, the enantiomers of verapamil separated with a resolution of 0.45 observed at a mole fraction of 0.30. The effect of methanol as a mobile phase modifier on the resolution obtained for the enantiomers of verapamil for solutions of these surfactants is shown in Figure 5-2. The resolution of the verapamil enantiomers increases as the percentage of methanol in the mobile phase increases in all four solutions up to 15 percent methanol. For a mole fraction of 0.30, the resolution increases to 25 percent methanol. A resolution of 0.55 was obtained for the verapamil enantiomers using a 50mM NaDC solution containing 25 percent methanol. A resolution of 1.5 was obtained for the verapamil enantiomers, using a solution of NaDC and $C_{12}E_4$, consisting of a mole fraction of 0.30 $C_{12}E_4$ and 25 percent methanol.

The effect of methanol on the enantiomeric separation of bi-2-naphthol was also investigated. The plots given in Figure 5-5 show the resolution obtained for the enantiomers of bi-2-naphthol versus the percentage of methanol in the solutions. The results are presented for 50mM solutions of NaDC and 50mM solutions of NaDC in mixtures with each of the three ethers. A resolution of 2.9 was observed for the bi-2-naphthol enantiomers using a NaDC in a solution containing 25 percent methanol. The use of mixed micellar solutions with $C_{12}E_4$ and NaDC, with and without methanol, decreased the resolution observed for the bi-2-naphthol enantiomers relative to solutions of NaDC alone.

The electropherograms given in Figures 5-9 and 5-10 clearly show the effect that adding $C_{12}E_4$ has on the chiral resolution observed for the enantiomers of verapamil and bi-2-naphthol. An increase in resolution is observed for the verapamil enantiomers while a decrease

in resolution is observed for the bi-2-naphthol enantiomers.

5.4.2 Evaluation of mobile phases containing $C_{12}E_6$ and $C_{10}E_8$

Experiments were performed to determine if other closely related ethers have similar effects on the chiral resolution of verapamil and related compounds. Two additional ethers having longer ether chain components were evaluated. The commercially available polyoxyethylene ethers, $C_{10}E_8$ and $C_{12}E_6$, were chosen for these studies. The experiments performed for the $C_{12}E_4$ containing mobile phases were repeated using $C_{10}E_8$ and $C_{12}E_6$.

Four plots are given in Figure 5-3 showing the observed enantiomeric resolution of verapamil versus methanol percentage for solutions of NaDC and three mixed micellar ratios with $C_{12}E_6$. The three levels of $C_{12}E_6$ investigated were mole fractions of 0.2, 0.3 and 0.4. No enhancement in the enantiomeric resolution of verapamil was obtained using solutions containing NaDC and $C_{12}E_6$ relative to solutions containing NaDC alone. Increased resolution is obtained with solutions containing NaDC, $C_{12}E_6$ and methanol compared to solutions containing NaDC and methanol. In the $C_{12}E_6$ study, the highest resolution of 0.8 for the enantiomers of verapamil was observed using a mobile phase consisting of mole fraction $C_{12}E_6$ of 0.3 and 15 percent methanol. The same trend in results was observed using solutions containing $C_{12}E_4$.

Four plots are given in Figure 5-4 showing the observed enantiomeric resolution of verapamil versus methanol percentage for solutions of NaDC and three mixed micellar ratios with $C_{10}E_8$. The three levels of $C_{10}E_8$ investigated were mole fractions of 0.1, 0.2 and 0.3. A slight enhancement in the resolution of the verapamil enantiomers is observed for solutions containing mole fractions $C_{10}E_8$ of 0.1 and 0.2 with 15 percent methanol, relative to solutions

containing NaDC with 15 percent methanol. The resolution obtained for the verapamil enantiomers using solutions containing $C_{10}E_8$, with and without added methanol, were consistently lower than the resolution obtained using solutions containing $C_{12}E_4$ or $C_{12}E_6$ under similar conditions.

The plots given in Figures 5-6 and 5-7 show the resolution of the enantiomers of bi-2-naphthol versus the percentage of methanol in the solutions. The results are presented for 50mM solutions of NaDC and 50mM solutions of NaDC in mixtures with each of the three ethers. The resolution obtained for the bi-2-naphthol enantiomers using solutions containing $C_{10}E_8$ and $C_{12}E_6$, with and without added methanol, were consistently lower than the resolution obtained using solutions containing NaDC.

5.5 Conclusions

Mixed micelles have the potential to provide an infinitely variable pseudostationary phase in micellar electrokinetic capillary chromatography. Studies have shown that bile salt solutions are capable of providing chiral resolution for some enantiomeric pairs. It is known that binary solutions of polyoxyethylene ethers and bile salts result in mixed micelles with larger aggregation numbers and decreased hydrophobicity of the micellar interior relative to solutions of bile salt alone. Thus, one approach to varying the pseudostationary phase to enhance the separation of less hydrophobic compounds is to use mixtures of the bile salts and one of the commercially available polyoxyethylene ethers. The ability of solutions of sodium deoxycholate and polyoxyethylene ethers to provide the desired enantiomeric separations was carefully evaluated by varying mole fraction composition of each ether and percentage of methanol in the 50mM surfactant solutions. These mixed micellar conditions were found to

increase the observed resolution for verapamil from 0.5 to 1.5. The solution which was found to provide the best separation for the verapamil enantiomers also provided chiral separations of closely related compounds, norverapamil and gallopamil. Thus, using a mobile phase containing NaDC, $C_{12}E_4$ and methanol, the simultaneous baseline separation of the enantiomers of verapamil, norverapamil and gallopamil was achieved.

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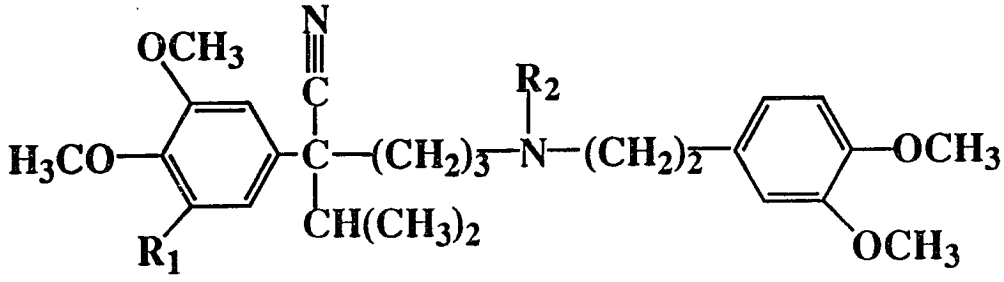
Table 5.1. Solution Conditions Investigated for the Chiral Resolution of ±Bi-2-naphthol and ±Verapamil: Type of Ether, Mole Fraction of Ether, and Percent Methanol.

	mole fraction ether			
% MeOH	0.1	0.2	0.3	0.4
0	$C_{10}E_8$	$C_{10}E_8$* $C_{12}E_6$ $C_{12}E_4$*	$C_{10}E_8$ $C_{12}E_6$* $C_{12}E_4$*	 $C_{12}E_6$ $C_{12}E_4$*
5	$C_{10}E_8$	$C_{10}E_8$* $C_{12}E_6$ $C_{12}E_4$	$C_{10}E_8$ $C_{12}E_6$ $C_{12}E_4$*	 $C_{12}E_6$ $C_{12}E_4$
15	$C_{10}E_8$*	$C_{10}E_8$* $C_{12}E_6$* $C_{12}E_4$*	$C_{10}E_8$* $C_{12}E_6$* $C_{12}E_4$*	 $C_{12}E_6$ $C_{12}E_4$*
25	$C_{10}E_8$	$C_{10}E_8$* $C_{12}E_6$ $C_{12}E_4$	$C_{10}E_8$ $C_{12}E_6$ $C_{12}E_4$*	 $C_{12}E_6$ $C_{12}E_4$

* indicates experiments which were duplicated on the HPCE system

Figure 5-1. Structures of Verapamil and Related Compounds



	R_1	R_2
verapamil	-H	$-CH_3$
norverapamil	-H	-H
gallopamil	$-OCH_3$	$-CH_3$

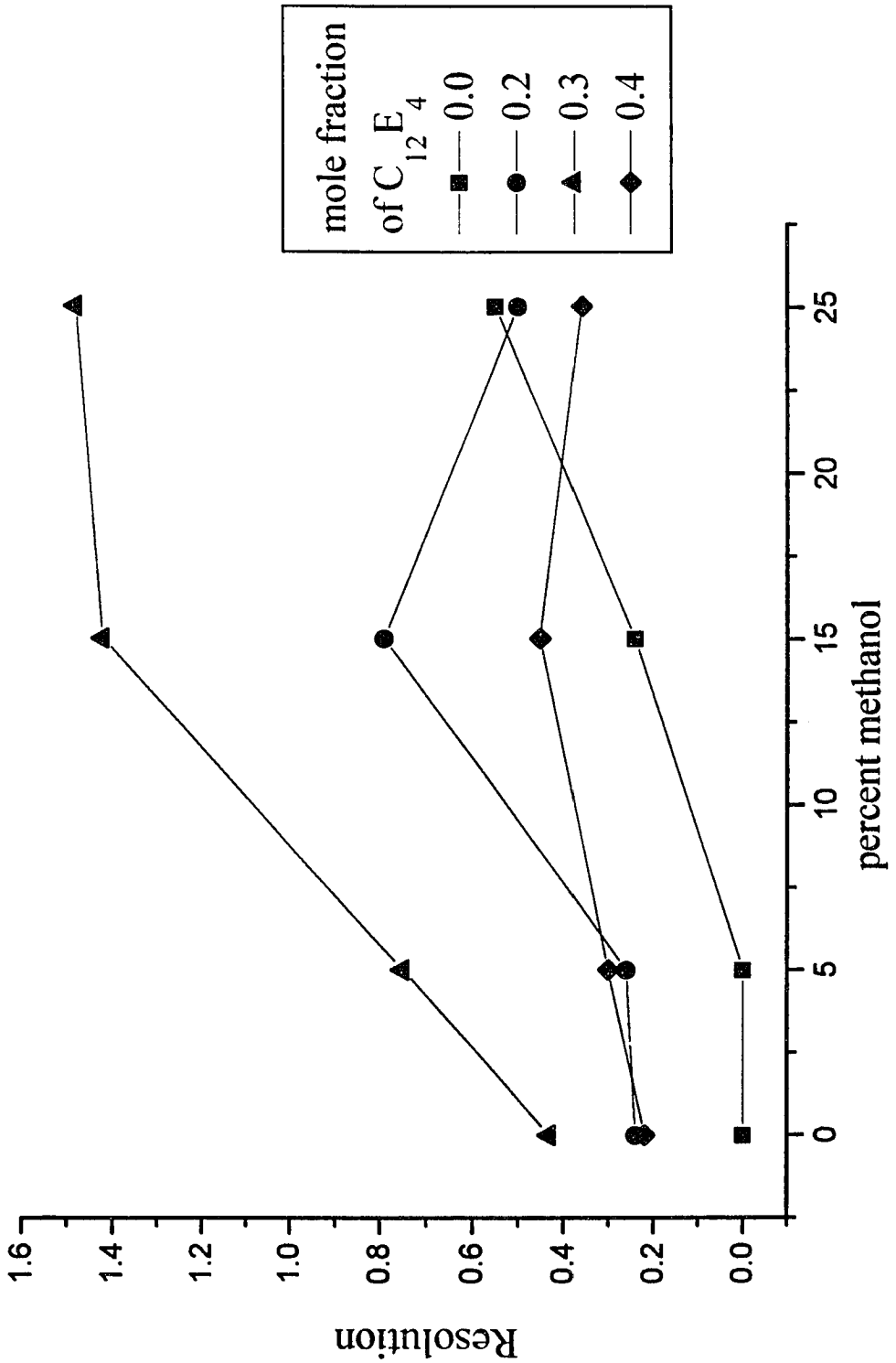


Figure 5-2. Observed enantiomeric resolution of verapamil at four mole fractions of $C_{12}E_4$ with increasing methanol percentage in a total surfactant concentration of 50mM for the NaDC/$C_{12}E_4$ mixed system.

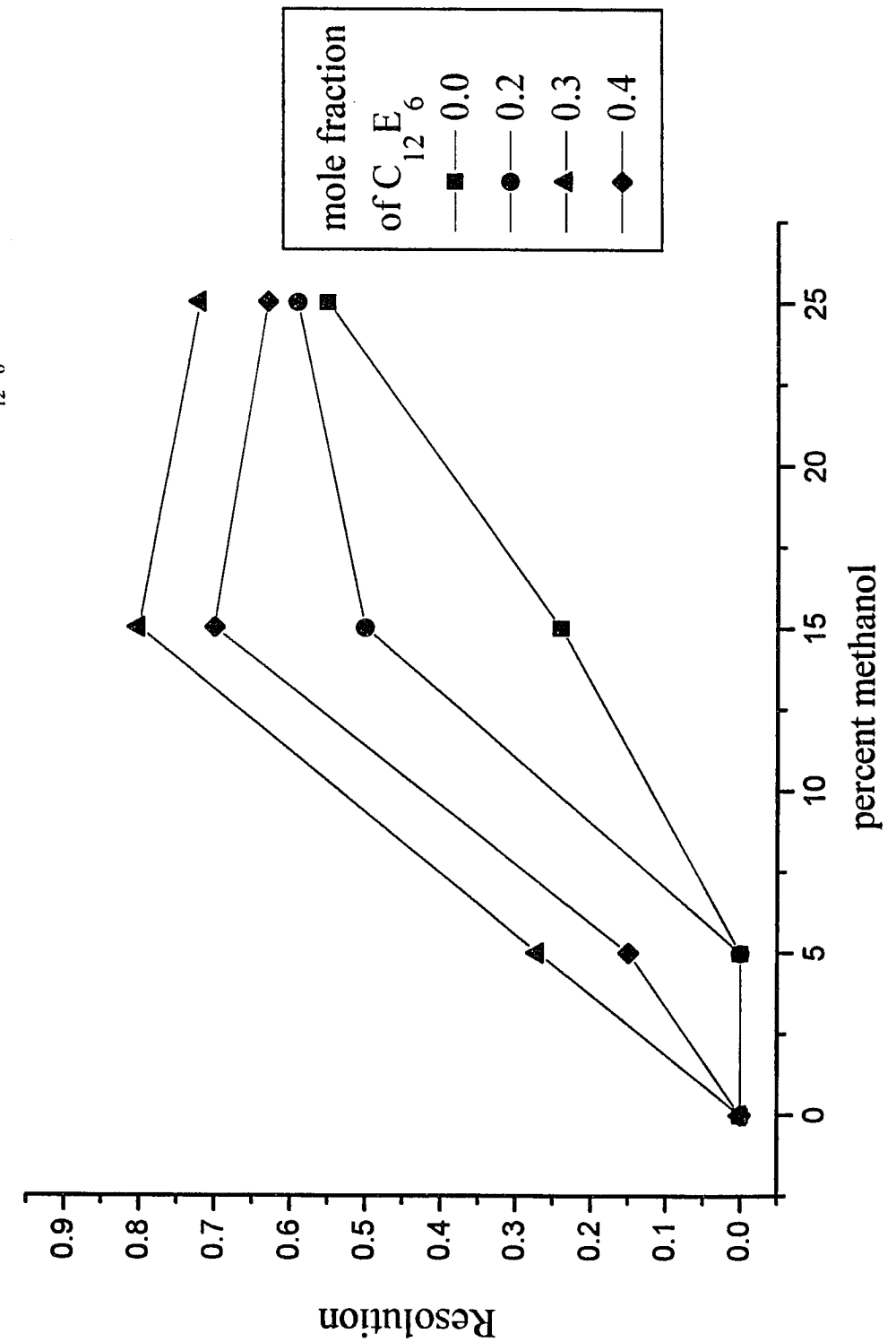


Figure 5-3. Observed enantiomeric resolution of verapamil at four mole fractions of $C_{12}E_6$ with increasing methanol percentage in a total surfactant concentration of 50mM for the NaDC/$C_{12}E_6$ mixed system.

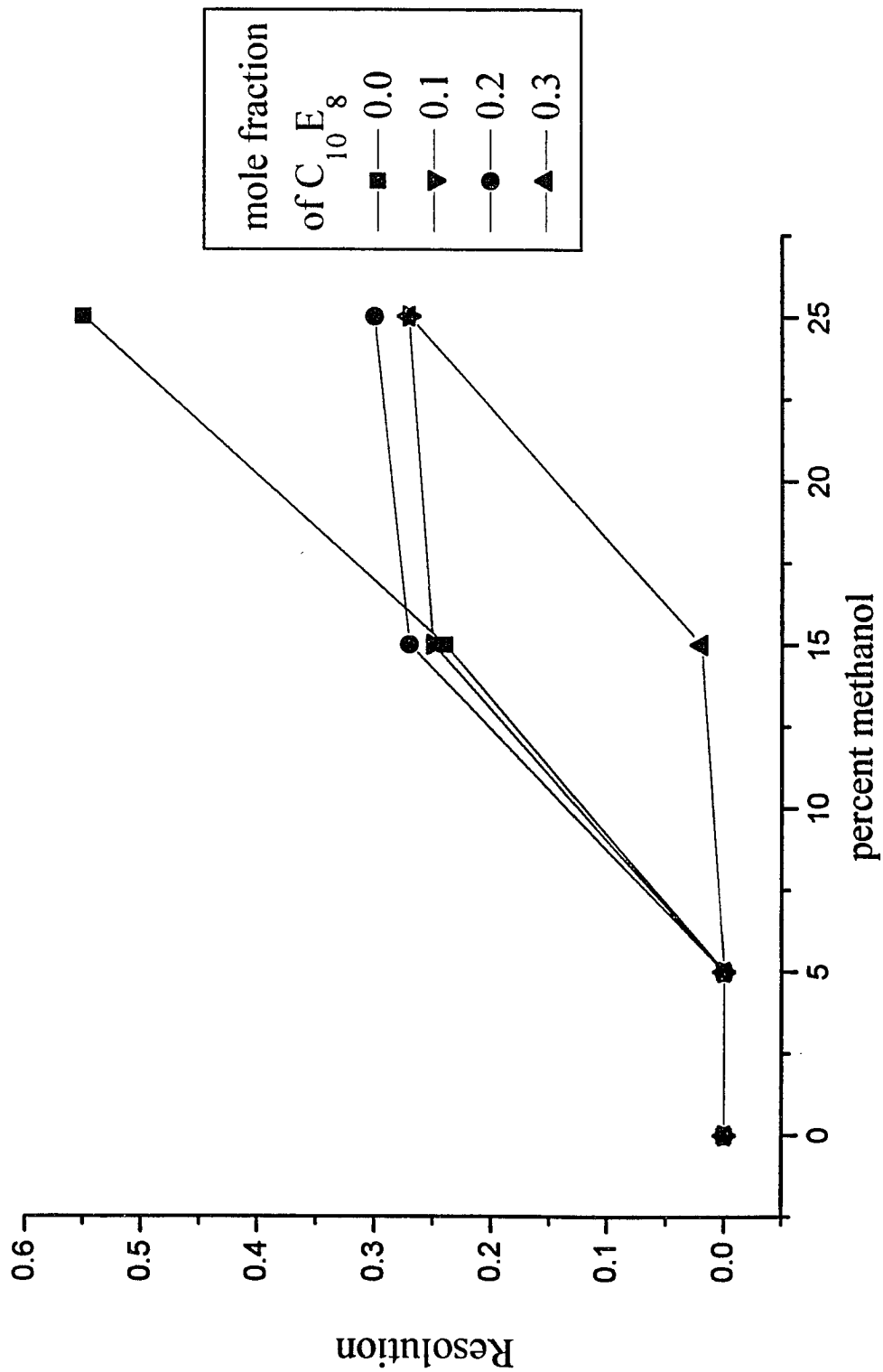


Figure 5-4. Observed enantiomeric resolution of verapamil at four mole fractions of $C_{10}E_8$ with increasing methanol percentage in a total surfactant concentration of 50mM for the NaDC/$C_{10}E_8$ mixed system.

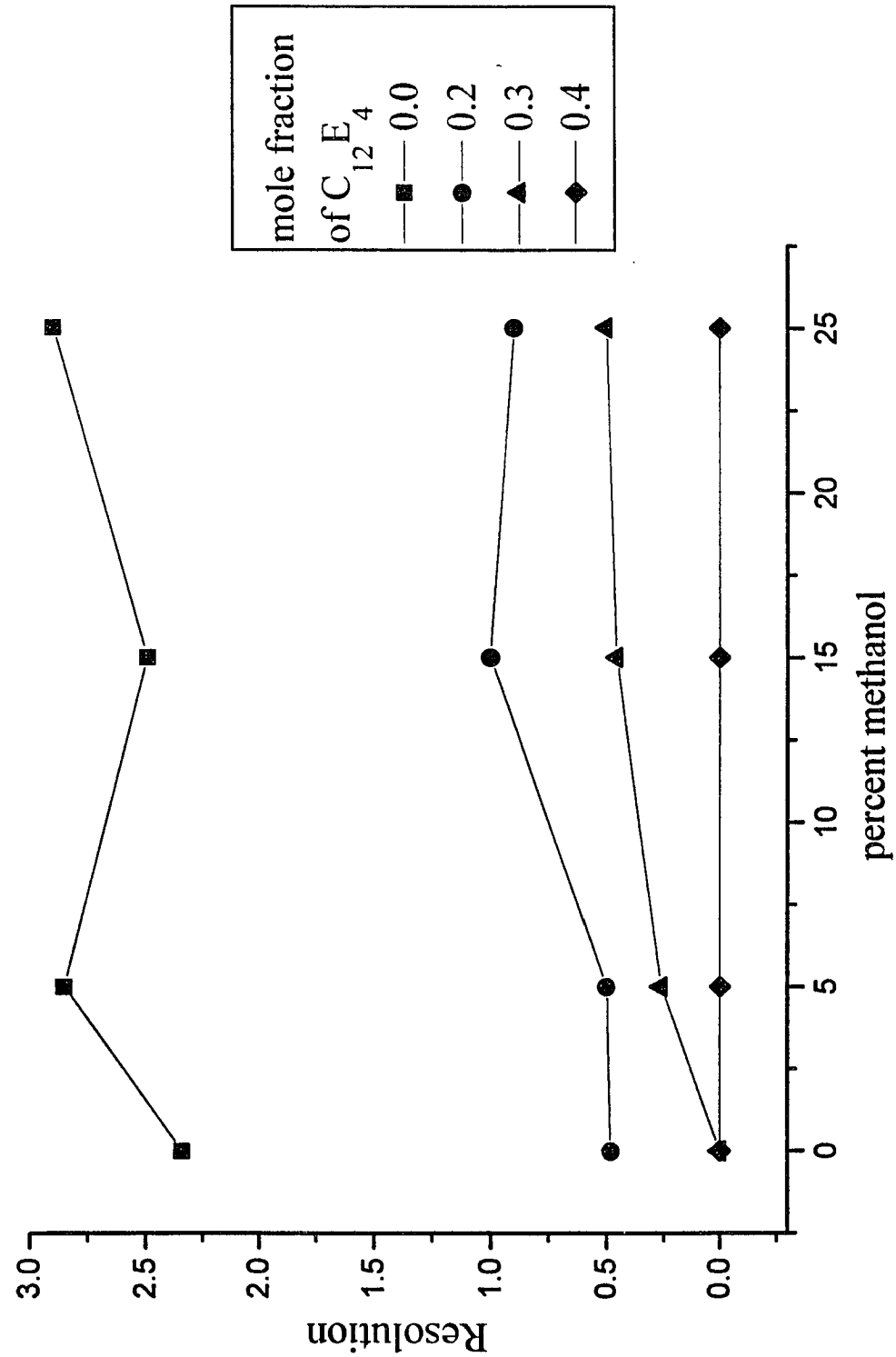


Figure 5-5. Observed enantiomeric resolution of bi-2-naphthol at four mole fractions of $C_{12}E_4$ with increasing methanol percentage in a total surfactant concentration of 50mM for the NaDC/$C_{12}E_4$ mixed system.

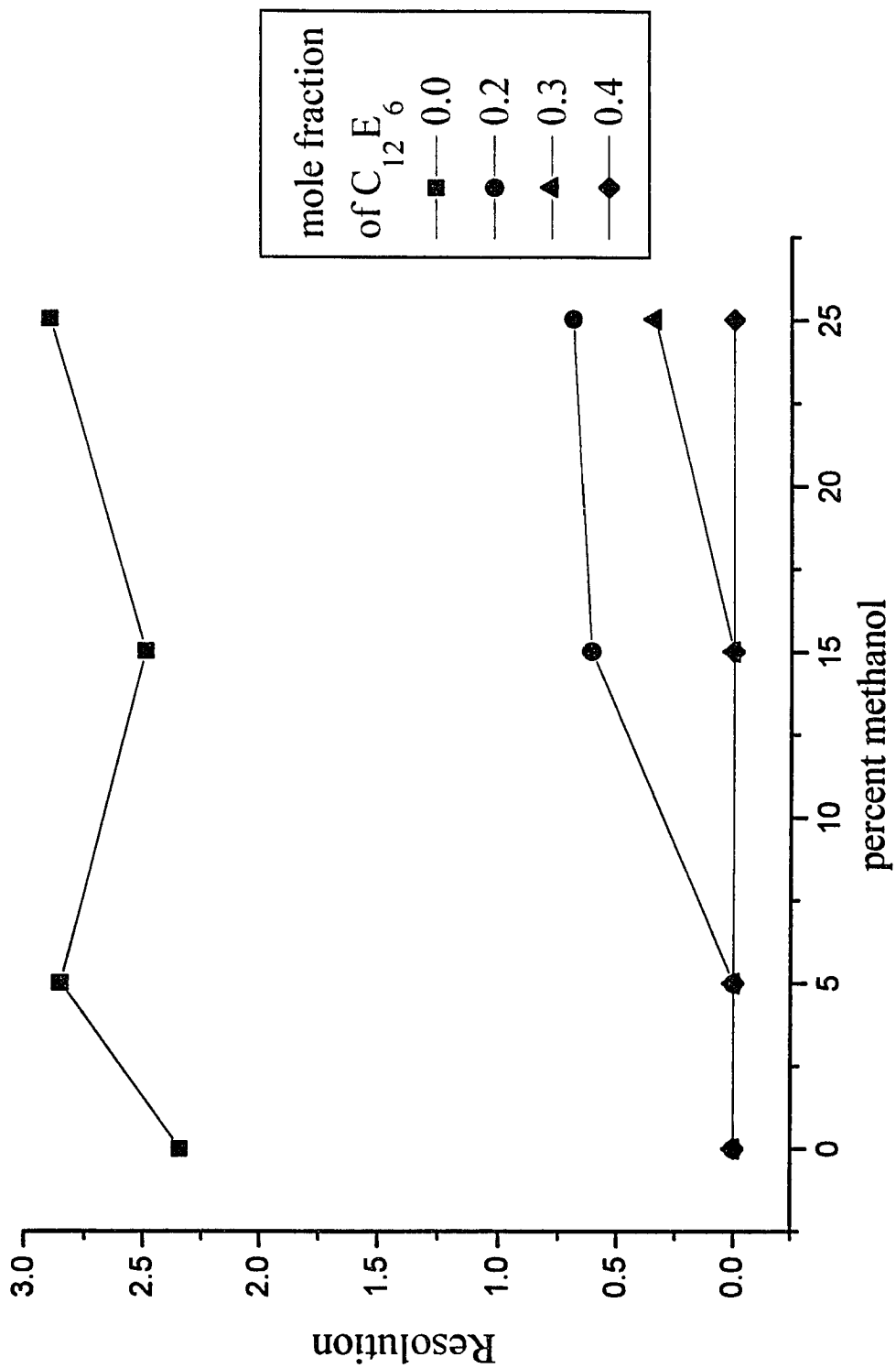


Figure 5-6. Observed enantiomeric resolution of bi-2-naphthol at four mole fractions of $C_{12}E_6$ with increasing methanol percentage in a total surfactant concentration of 50mM for the NaDC/$C_{12}E_6$ mixed system.

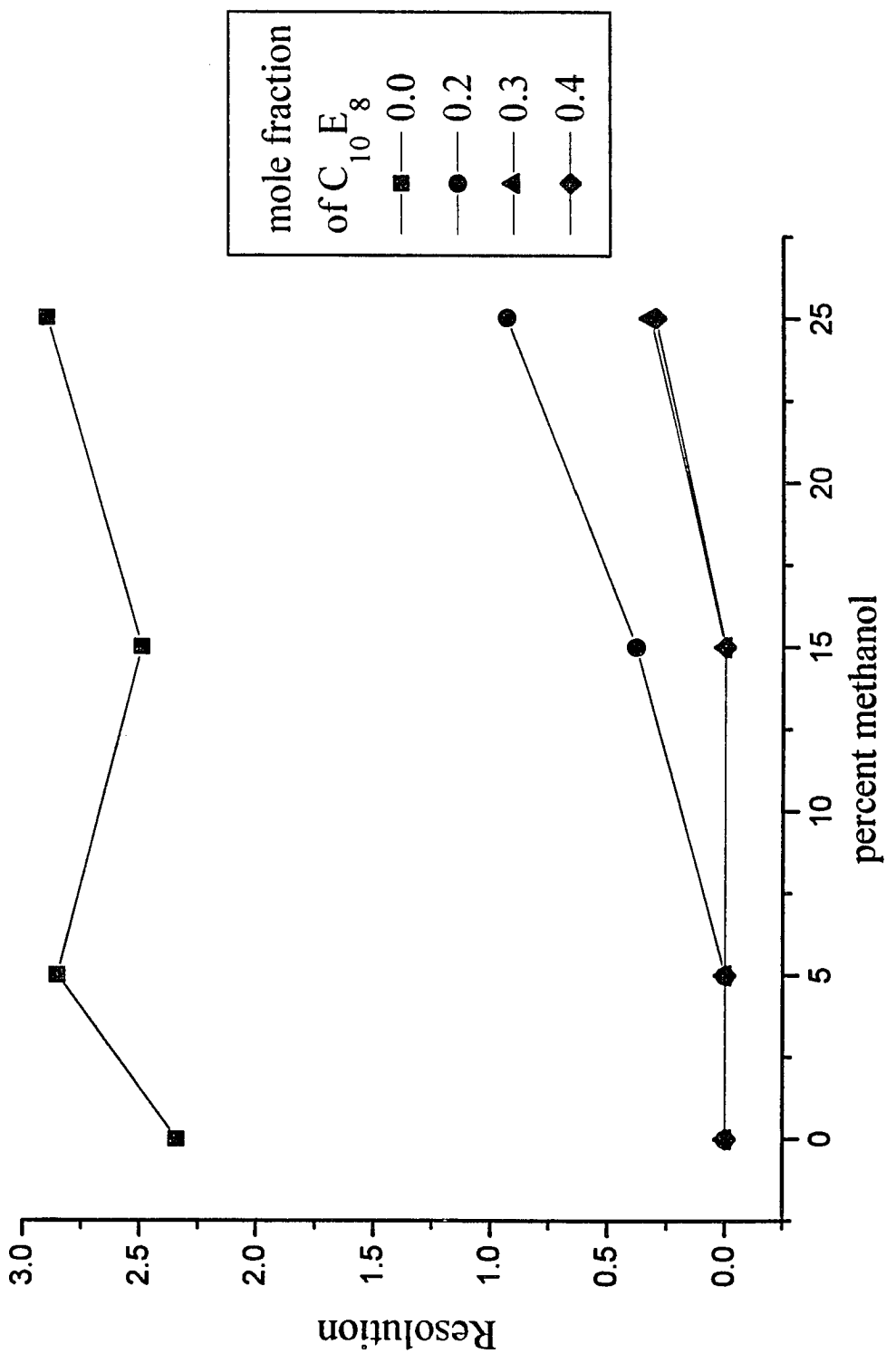


Figure 5-7. Observed enantiomeric resolution of bi-2-naphthol at four mole fractions of $C_{10}E_8$ with increasing methanol percentage in a total surfactant concentration of 50mM for the NaDC/$C_{10}E_8$ mixed system.

Figure 5-8. Capillary electropherogram of racemic verapamil, norverapamil and gallopamil using a mixed surfactant solution of NaDC and $C_{12}E_4$. The solution contained $C_{12}E_4$ mole fraction of 0.3, 25% methanol, and a total surfactant concentration of 50mM. The solution injected contained each enantiomer at a concentration of 0.25 mg/mL in methanol. Applied voltage 20kV. Absorbance at 210nm. Observed average current of 8.4 microamps. Acquired using the laboratory assembled CE instrument. Peak identifications are as follows:
1) (-)-gallopamil, 2) (+)-gallopamil, 3) (-)-verapamil, 4) (+)-verapamil, 5) (-)-norverapamil, and 6) (+)-norverapamil.

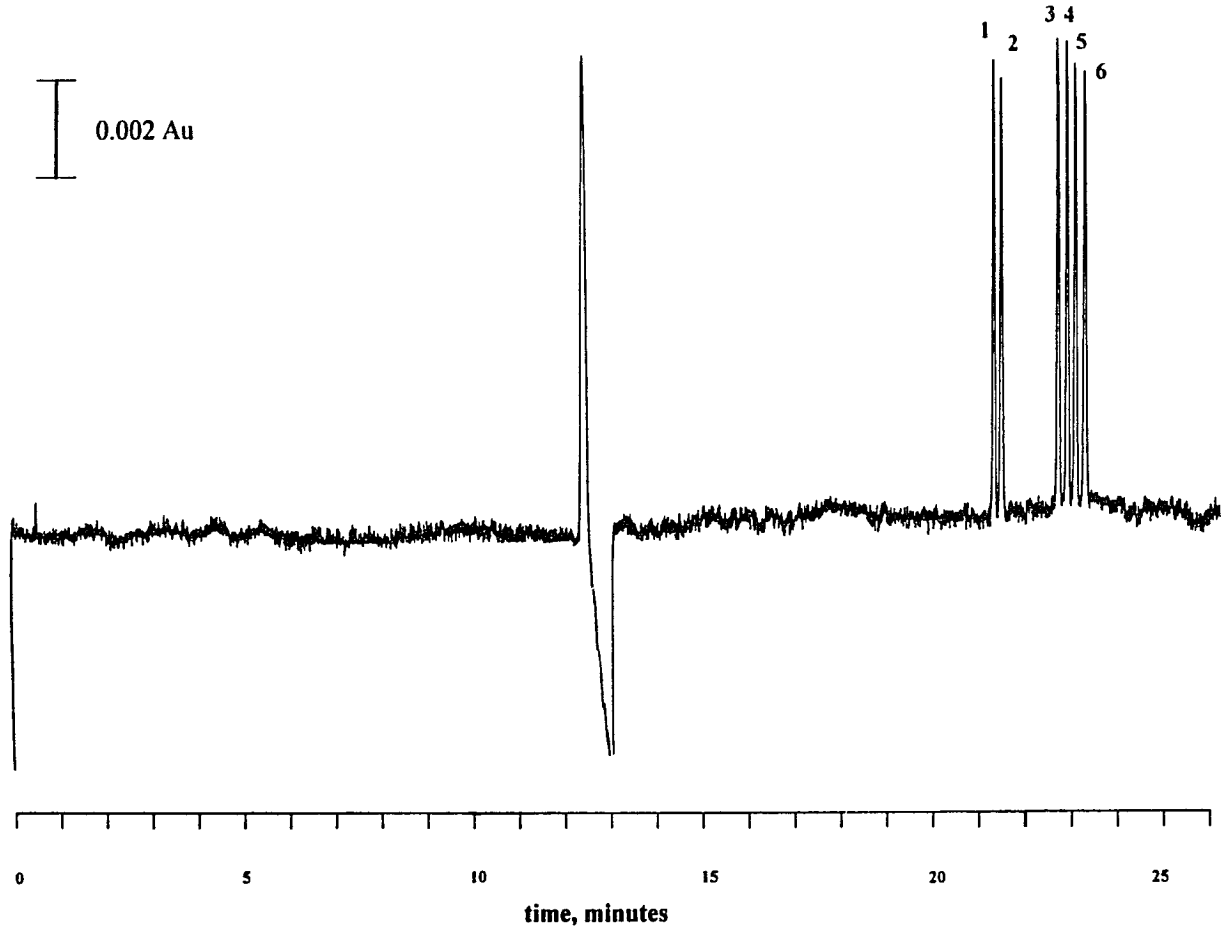


Figure 5-9. Capillary electropherogram of racemic verapamil and bi-2-naphthol using a mixed surfactant solution of NaDC and $C_{12}E_4$. The solution contained $C_{12}E_4$ mole fraction 0.3, 25% methanol at a total surfactant concentration of 50mM. Applied voltage 20kV. Observed current 22.5μA. Absorbance at 210nm for the complete electropherogram. Inset absorbance at 200nm from 18 to 23.2mins. Acquired using the HPCE system. Peak identifications are as follows: 18.625min, (-)-verapamil; 18.827, (+)-verapamil; 22.207, (+)-bi-2-naphthol; 22.315, (-)-bi-2-naphthol.

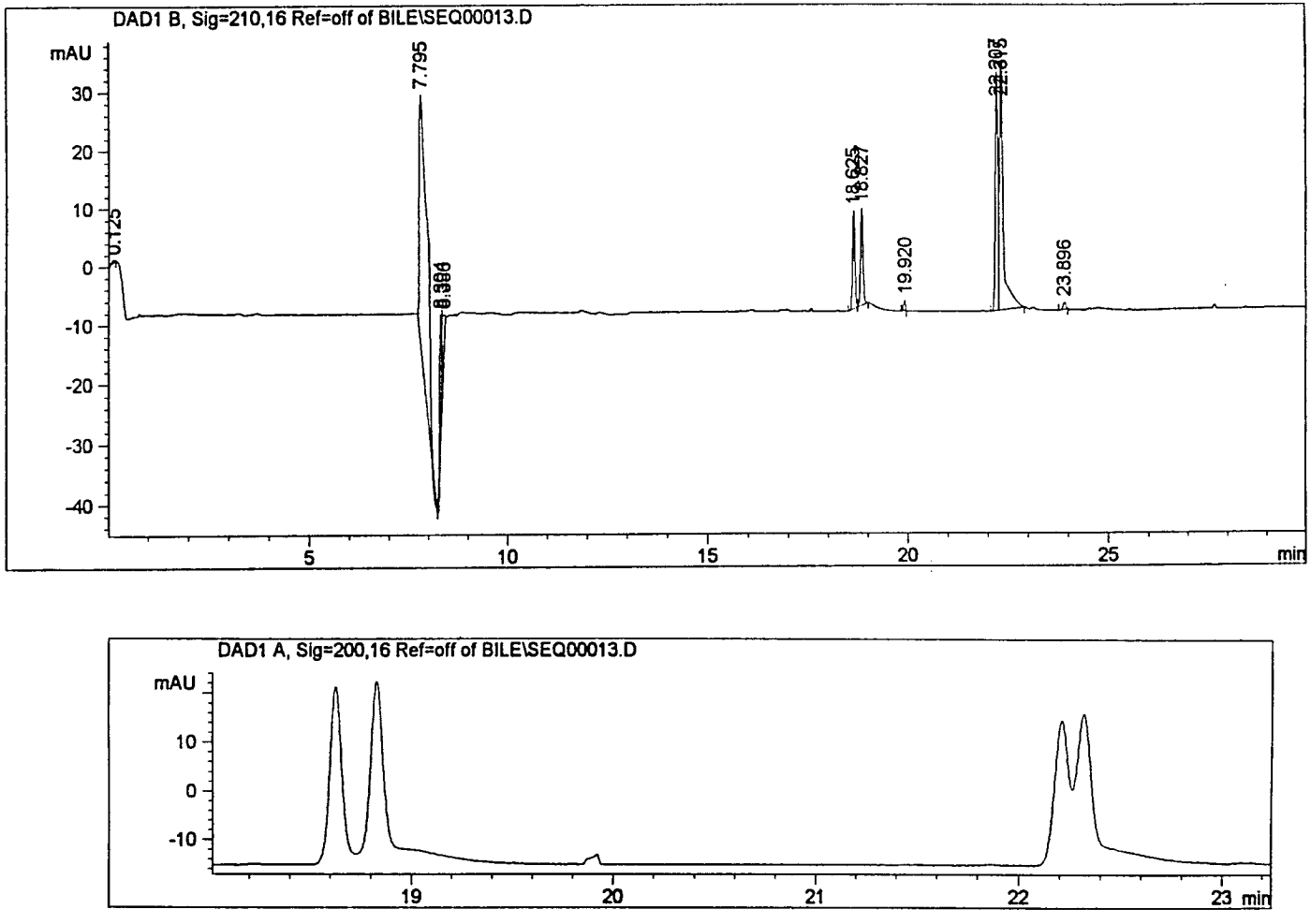
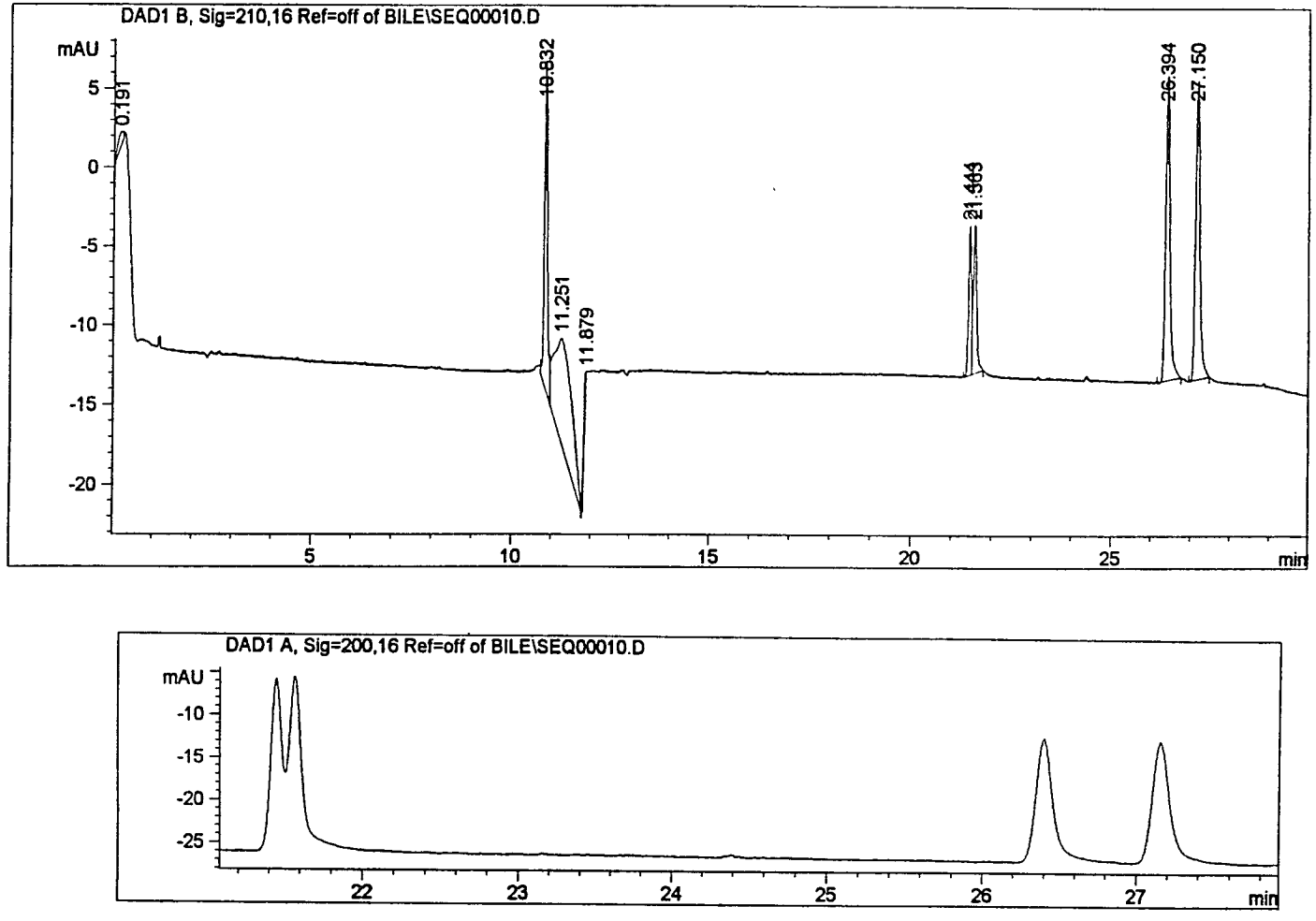


Figure 5-10. Capillary electropherogram of racemic verapamil and bi-2-naphthol using a solution of NaDC. The solution contained 25% methanol and a total surfactant concentration of 50mM. Applied voltage 20kV. Observed current 23.5μA. Absorbance at 210nm for the complete electropherogram. Inset absorbance at 200nm from 21 to 28mins. Acquired using the HPCE system. Peak identifications are as follows: 21.444min, (-)-verapamil; 21.583, (+)-verapamil; 26.394, (+)-bi-2-naphthol; 27.150, (-)-bi-2-naphthol.



CHAPTER 6

CORRELATIONS BETWEEN MICELLE HYDROPHOBICITY AND CHIRAL RESOLUTION

6.1. Introduction

The addition of polyoxyethylene ethers to bile salt solutions was shown in Chapter 5 to improve chiral resolution for verapamil and related compounds in micellar electrokinetic capillary chromatography (MECC). Mixtures of one bile salt, sodium deoxycholate, and three polyoxyethylene ethers, polyoxyethylene-4-dodecyl ether, polyoxyethylene-6-dodecyl ether and polyoxyethylene-8-decyl ether, were previously investigated. The greatest increase in chiral resolution was observed using polyoxyethylene-4-dodecyl ether ($C_{12}E_4$). Further studies have been conducted with solutions containing $C_{12}E_4$ in binary mixtures with sodium deoxycholate (NaDC) and sodium cholate (NaC). These mixed surfactant solutions have been evaluated as chiral pseudophases in MECC. The results of these studies have indicated the type of racemic mixtures which can be resolved using solutions of bile salts and polyoxyethylene ethers. Fluorescence spectroscopy using pyrene as a probe molecule has provided information on the relative hydrophobicities of these mixed surfactant solutions. Studies of these mixed micellar solutions indicate that correlations exist between micellar hydrophobicity, as determined by fluorescence studies, and chiral resolution. These correlations have allowed the prediction of the appropriate mole fraction ether, which will provide chiral resolution for a given enantiomeric pair, when comparing some solutions containing mixtures of bile salts and ethers.

The aggregation behavior of the bile salts under various conditions has been studied

using light scattering (1,2), nuclear magnetic resonance, electron spin resonance and x-ray techniques (3-5). From the results of these studies, a helical model has been proposed in which the hydroxyl groups of the steroid ring backbone are oriented towards the core of the micelle (3-5). This model may be important for the understanding of chiral recognition in a bile salt micelle. The number and orientation of the hydroxyl groups will change the possibilities for chiral interactions inside the core of the bile salt micelle. The studies involving different bile salts have shown that the number and orientation of the hydroxyl groups does affect chiral recognition for the compounds investigated here by MECC.

The relative hydrophobicities of aggregates in micellar solutions have been correlated to spectral data using pyrene as a probe molecule (6-10). The emission spectrum of pyrene in aqueous micellar solutions has a considerable fine structure. Five distinct fluorescence emission maxima in the spectrum are observed between 350 and 400 nm using an excitation wavelength of 330 nm. Relative hydrophobicity measurements have been reported based on the ratio of the intensities of the first to third fluorescence peaks (6-10). These reports have described a general method for determining the hydrophobicity of detergent solutions (6-8) and several bile salt solutions(9,10).

The properties of mixed micelles formed in solutions of bile salts and polyoxyethylene ethers have been investigated by several techniques (11-15). The addition of polyoxyethylene-8-decyl ether ($C_{10}E_8$) in various mole ratios to NaDC has been shown to alter the critical micellar concentration (CMC), the aggregation number (AN) of the micelles, and the ability of the micelles to solubilize cholesterol. For example, a mixed solution of the $C_{10}E_8$ ether and NaDC containing an ether mole fraction of 0.25, was determined to have a CMC of 1.63 mM

and an AN of 25. A solution containing NaDC was determined to have a CMC of 3.16 mM and an AN of 18 under the same experimental conditions (15). Asano and coworkers indicate that the hydrophobicity of the interior of the mixed micelle decreases with increased mole fraction of ether (15). For example, increasing the mole fraction of ether from 0.00 to 0.43 in mixed solutions of NaDC and $C_{10}E_8$ results in the formation of micelles which decreased in hydrophobicity. A significant increase in the solubilization of cholesterol was observed for mixed solutions of NaDC and $C_{10}E_8$ when the mole fraction of ether was increased from 0.00 to 0.40. The addition of polyoxyethylene ethers to bile salt solutions can be used to create a series of micellar solutions with decreasing hydrophobicities. These solutions can be used as a variable pseudophase in MECC.

The compounds previously investigated in Chapter 5 (verapamil, norverapamil, gallopamil and bi-2-naphthol) were further investigated here by MECC. BAYK8644 is a chiral compound under development as a cardiac drug by the Bayer Corporation (16). All five of these compounds were used as chiral test analytes to investigate the relationship between micelle hydrophobicity, as indicated by fluorescence measurements, and chiral resolution obtained by MECC.

6.2. Experimental

6.2.1. Apparatus for CE

Studies were conducted using the laboratory assembled capillary electrophoresis instrument previously described. The column used for these studies was a bare fused silica capillary, 50 micron ID, 350 micron OD (Supelco, Bellefonte, PA). The detection wavelength used was 210nm.

6.2.2. Materials

Sodium deoxycholate (NaDC) and sodium cholate (NaC) were purchased from Aldrich Chemical Co. (Milwaukee, WI). $C_{12}E_4$, bi-2-naphthol enantiomers, and pyrene were purchased from Aldrich. The R and S enantiomers of verapamil hydrochloride, methoxyverapamil hydrochloride (gallopamil hydrochloride) and BAYK8644 were purchased from Research Biochemicals Inc. (Natick, MA.). The R and S enantiomers of norverapamil were kindly provided by Dr. L. Miller (Searle Chemical Sciences Department, Skokie, IL). Reagent grade sodium hydroxide and sodium chloride and HPLC grade methanol were purchased from Fisher Scientific (Pittsburgh, PA). Deionized, distilled water was used for the preparation of all solutions.

6.2.3. Solution Preparation

Surfactant solutions were prepared by mixing appropriate volumes of stock solutions containing each type of surfactant. The solutions were prepared containing methanol in the indicated percentages by volume. Polyoxyethylene ether stock solutions were prepared by dissolving the appropriate quantity of the surfactant in water to achieve a final surfactant concentration of 50 mM. Stock solutions of each bile salt at 100 mM were prepared by dissolving the appropriate quantity of the bile salt in water. Mixed micellar solutions were prepared at a total surfactant concentration of 50mM by combining appropriate volumes of bile salt stock solution, polyoxyethylene ether stock solution, methanol and water. The pH of the resulting solutions was between 8.1 and 8.3. Methanol solutions containing the test analytes; verapamil, norverapamil, gallopamil, bi-2-naphthol and BAYK8644 were prepared from the individual enantiomers. The injected concentration of each enantiomer was between

0.25 and 1.0 mg/mL.

6.2.4. Fluorescence Studies

Pyrene was used as a probe to determine the relative hydrophobicities of the micelles using a method previously reported for mixtures of bile salts and polyoxyethylene ethers (6-10). The fluorescence emission spectrum of pyrene in the micellar solutions was measured from 350 nm to 500 nm using an excitation wavelength of 333 nm. The pyrene concentration used for these experiments was $1X10^{-5}$ M. The spectral data were acquired using an SLM Aminco Bowman Series 2 Luminescence Spectrometer (Rochester, NY). The emission bandpass and scan rate were set to 0.5 nm and 0.5 nm/s, respectively.

6.2.5. CE Experimental Technique

Prior to performing an experiment, the capillary was rinsed with a 0.1 M sodium hydroxide solution for two minutes. The surfactant solution used for the experiment was rinsed through the capillary for an additional 2 minutes by applying pressure to a vial containing the solution on the anode side of the capillary. Injection of analytes was performed hydrodynamically by raising the cathode end of the column to a height of 15 cm above the anode for 1 to 6 seconds.

The capillary used had an overall length of 75 cm with a length of 65 cm to detection. Typical experimental runs involved rinsing the column with the sodium hydroxide solution and water, followed by the surfactant solution, and injecting the solution containing the solute(s) of interest and then simultaneously switching the voltage on and starting the data collection. The resolution reported is based on baseline width measurements determined from the strip chart recordings of the electropherograms.

6.2.6. Experimental Conditions Investigated Using CE

The type of bile salt and mole fractions of ether used for the separation of each solute are given in Table 6-1.

6.3. Results

6.3.1. Fluorescence Experiments

Solutions containing binary mixtures of polyoxyethylene-4-dodecyl ether ($C_{12}E_4$) and each of two bile salts were investigated by acquiring fluorescence spectra using pyrene as a probe. The two bile salts studied were NaC and NaDC. The emission spectra of pyrene in a solution of 50mM NaDC and 50mM $C_{12}E_4$ are given in Figures 6-1 and 6-2, respectively. The emission spectrum of pyrene in a mixed surfactant solution containing a mole fraction of 0.25 ether is given in Figure 6-3.

Solutions containing binary mixtures of $C_{12}E_4$ with NaC and $C_{12}E_4$ with NaDC were investigated using fluorescence with pyrene as the probe molecule. Measurements of the ratios of the first to third vibronic band intensities were made from the fluorescence spectra. Figure 6-4 contains a plot of these ratios versus mole fraction bile salt in the mixed solution.

6.3.2. MECC Separations using mixed micelles of Bile Salts and $C_{12}E_4$

Figure 6-5 is a plot of the chiral resolution obtained by MECC for the enantiomers of the test compounds using solutions of NaC and binary mixtures of NaC and $C_{12}E_4$. Solutions of NaC and binary mixtures of NaC with $C_{12}E_4$ at mole fractions of 0.10, 0.20, 0.25 and 0.40 were used throughout the study. Experiments were performed for the following compounds: verapamil, norverapamil, gallopamil, bi-2-naphthol, and BAYK8644. The total surfactant concentration was 50mM and the pH was between 8.0 and 8.2 for all solutions. Capillary

electropherograms of the chiral separations of a mixture of two solutes, verapamil and bi-2-naphthol, using solutions containing NaC and a mixture of NaC and $C_{12}E_4$ are given in Figures 6-6 and 6-7, respectively. The chiral resolution of all compounds was severely degraded and not reproducible when methanol was added to solutions of NaC and NaC in mixtures with $C_{12}E_4$.

Figure 6-8 is a plot of the chiral resolution obtained by MECC for the enantiomers of the test compounds using solutions of NaDC and binary mixtures of NaDC and $C_{12}E_4$. Solutions of NaDC and binary mixtures of NaDC with $C_{12}E_4$ at mole fractions of 0.20, 0.30 and 0.40 were used throughout the study. Experiments were performed for the following compounds: verapamil, norverapamil, gallopamil, bi-2-naphthol, and BAYK8644. The total surfactant concentration was 50mM and the pH was between 8.0 and 8.2 for all solutions.

Figure 6-9 is a plot of the chiral resolution obtained by MECC for the enantiomers of the test compounds using solutions of NaDC and binary mixtures of NaDC and $C_{12}E_4$ containing 25% methanol. Solutions of NaDC with methanol and ternary mixtures of NaDC, methanol and $C_{12}E_4$ at mole fractions of 0.20, 0.30, and 0.40 were used throughout the study. Experiments were performed for the following compounds: verapamil, norverapamil, gallopamil and bi-2-naphthol. The total surfactant concentration was 50mM and the pH was between 8.0 and 8.2 for all solutions.

6.4. Discussion

The fluorescence emission spectra of pyrene in three different micellar environments is given Figures 6-1, 6-2 and 6-3. Figures 6-1 and 6-2 are plots of the fluorescence emission of pyrene observed in 50mM solutions of NaDC and $C_{12}E_4$. Figure 6-3 is a plot of the

fluorescence emission of pyrene observed in a mixed micellar solution of NaDC and $C_{12}E_4$ at a mole fraction of 0.25 of the ether and a total surfactant concentration of 50mM. A trend is observed in the ratios of the of the first to third vibronic band intensities, I_1/I_3, in these spectra. A decrease in the ratio, I_1/I_3, is observed from solutions of ether to mixed surfactants and from solutions of mixed surfactants to bile salt. Figure 6-4 is a plot of these fluorescence ratios versus mole fraction of bile salt which were determined from a series of binary mixtures of NaDC with $C_{12}E_4$ and NaC with $C_{12}E_4$. This decrease in the ratio is observed using both series of bile salt and ether mixed solutions. A region of these ratios which appears to have a linear relationship with mole fraction of bile salt in the mixed solution is observed in both series. This linear region is observed from 0.50 to 1.00 mole fraction of bile salt for the NaDC series. This linear region is observed from 0.60 to 1.00 mole fraction of bile salt for the NaC series. The magnitude of the ratios is consistently higher for the NaC series than for the NaDC series at the same mole fractions of bile salt.

This ratio of the fluorescence intensities observed for pyrene has been correlated to the hydrophobicity of the molecular environment (6-12). A lower fluorescence ratio indicates an environment of increased hydrophobicity. Thus, the hydrophobicites of the micelles formed are consistently lower for the NaC series than for the NaDC series at the same mole fractions of bile salt. A series of solutions of NaC and $C_{12}E_4$ containing mixed micelles with a steady decrease in hydrophobicity have been observed up to an ether mole fraction of 0.40. A series of solutions of NaDC and $C_{12}E_4$ containing mixed micelles with a steady decrease in hydrophobicity has been observed up to an ether mole fraction of 0.50. The data presented here for the mixed solutions of NaDC with $C_{12}E_4$ and NaC with $C_{12}E_4$ show the same trends

previously reported for mixed solutions of bile salts and the polyoxyethylene ether, $C_{10}E_8$ (11-15).

Figure 6-5 is a plot of the chiral resolution obtained for several solutes versus mole fraction of ether using solutions of NaC and a series of solutions containing mixtures of NaC with $C_{12}E_4$. The observed chiral resolution is higher for verapamil, norverapamil and gallopamil using solutions containing ether at mole fractions of 0.10, 0.20 and 0.25 compared to solutions containing ether at a mole fraction of 0.40 or solutions containing NaC alone. The observed chiral resolution decreases for bi-2-naphthol and BAYK8644 as the mole fraction of ether is increased. These changes in chiral resolution are observed for verapamil and bi-2-naphthol in the electropherograms in Figures 6-6 and 6-7 using solutions containing NaC, and a mixture of NaC with $C_{12}E_4$, respectively.

The experiments were repeated using solutions containing the bile salt, NaDC. Figure 6-8 is a plot of observed chiral resolution for several solutes versus mole fraction of ether using solutions of NaDC and a series of solutions containing mixtures of NaDC with $C_{12}E_4$. Chiral resolution is improved for verapamil, norverapamil and gallopamil using solutions containing ether at mole fractions of 0.20, 0.30 and 0.40 compared to solutions containing NaDC alone. The resolution of the enantiomers of bi-2-naphthol and BAYK8644 decreases as the mole fraction of ether increases.

The experiments were repeated using solutions containing NaDC with 25% methanol. Figure 6-9 is a plot of observed chiral resolution for several solutes versus mole fraction of ether using solutions of NaDC with 25% methanol and a series of solutions containing mixtures of NaC and $C_{12}E_4$ with 25% methanol. The observed chiral resolution is higher for

verapamil, norverapamil and gallopamil using solutions containing ether at a mole fraction of 0.30 compared to solutions containing ether at mole fractions of 0.20 and 0.40 or solutions containing NaC alone. The observed chiral resolution decreases for bi-2-naphthol as the mole fraction of ether is increased.

6.5. Conclusions

The highest chiral resolution observed for several compounds with each series of mixed micellar solutions corresponded to solutions containing micelles of similar hydrophobicity. The highest value for chiral resolution of verapamil was observed using an ether mole fraction of 0.3 in solutions containing mixtures of NaDC and $C_{12}E_4$. The highest values for chiral resolution of verapamil were observed using ether mole fractions of 0.1 and 0.2 in solutions containing mixtures of NaC and $C_{12}E_4$. Similar correlations can also be made for norverapmil and gallopamil. The fluorescence ratios corresponding to the highest values of chiral resolution for verapamil, norverapamil and gallopamil using mixed micellar solutions based on the bile salts NaDC and NaC are estimated at 0.76 and 0.82. The highest values for chiral resolution the enantiomers of bi-2-naphthol and BAYK8644 occurred using solutions containing micelles of the highest hydrophobicity. The results of these studies suggest that a given molecule will have the most favorable chiral interaction at a specific micellar hydrophobicity in bile salt MECC separations.

Additional work in the laboratory has been conducted using bile salt solutions of sodium chenodeoxycholate and sodium ursodeoxycholate, alone and in mixtures with polyoxyethylene ethers, for MECC separations (16). This additional work has supported the correlation between micellar hydrophobicity and chiral resolution by MECC for some

compounds. This work involving additional compounds has shown that larger, linear molecules have an increased chiral resolution with these mixed micelles, whereas smaller, compact solutes have better chiral interactions with the pure bile salts. These conclusions are supported by the work presented here.

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Table 6-1. The Type of Bile Salt and Mole Fractions of Ether Tested for the Chiral Separation of Each Solute.

	mole fraction ether				
	0.0	0.1	0.2	0.3	0.4
NaC	verapamil norverapamil gallopamil bi-2-naphthol Bay K8644	verapamil norverapamil gallopamil bi-2-naphthol Bay K8644	verapamil norverapamil gallopamil bi-2-naphthol Bay K8644	verapamil norverapamil gallopamil bi-2-naphthol Bay K8644	verapamil norverapamil gallopamil bi-2-naphthol Bay K8644
NaDC	verapamil norverapamil gallopamil bi-2-naphthol Bay K8644		verapamil norverapamil gallopamil bi-2-naphthol Bay K8644	verapamil norverapamil gallopamil bi-2-naphthol Bay K8644	verapamil norverapamil gallopamil bi-2-naphthol Bay K8644
NaDC with 25% MeOH	verapamil norverapamil gallopamil bi-2-naphthol		verapamil norverapamil gallopamil bi-2-naphthol	verapamil norverapamil gallopamil bi-2-naphthol	verapamil norverapamil gallopamil bi-2-naphthol

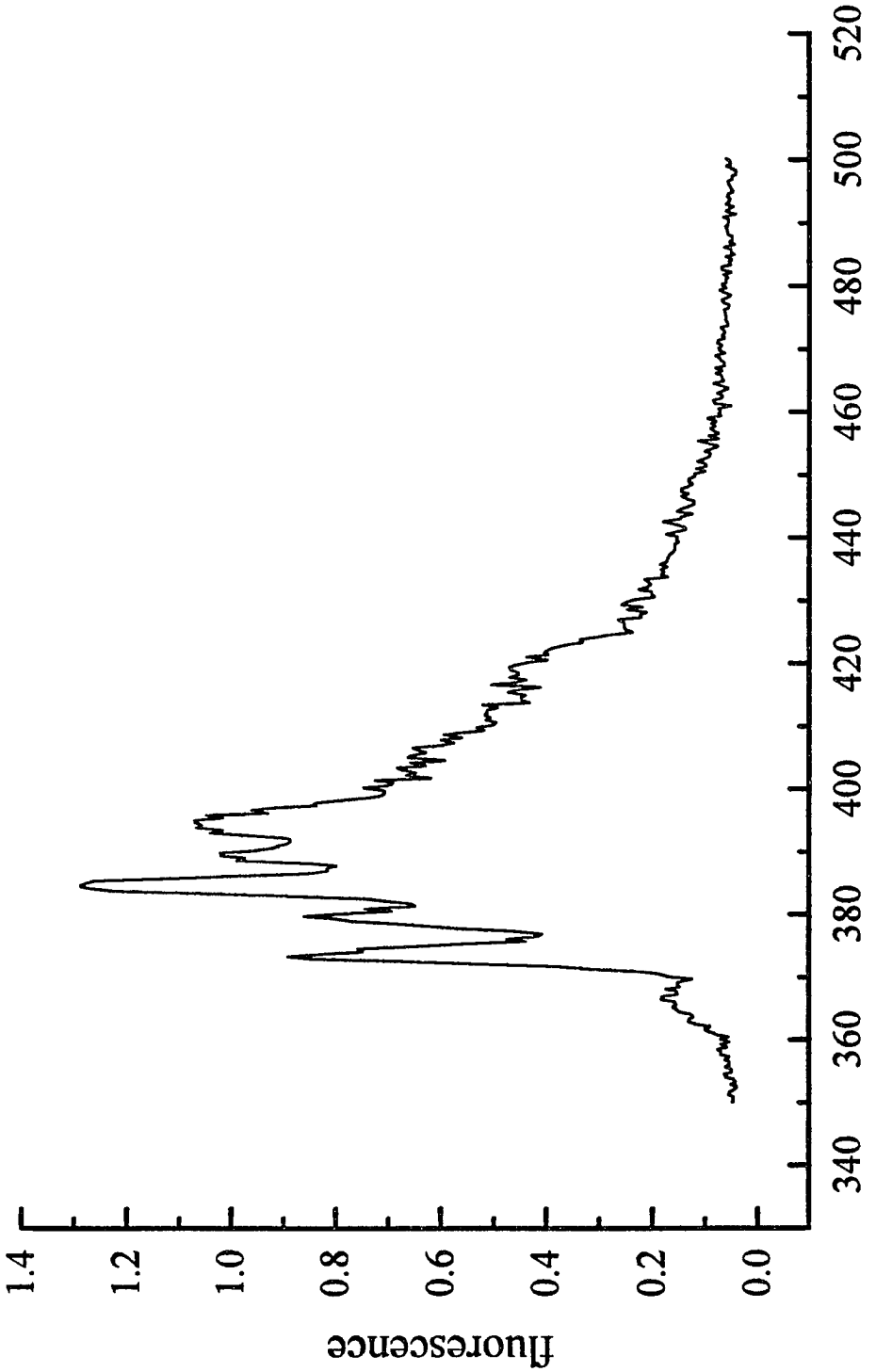
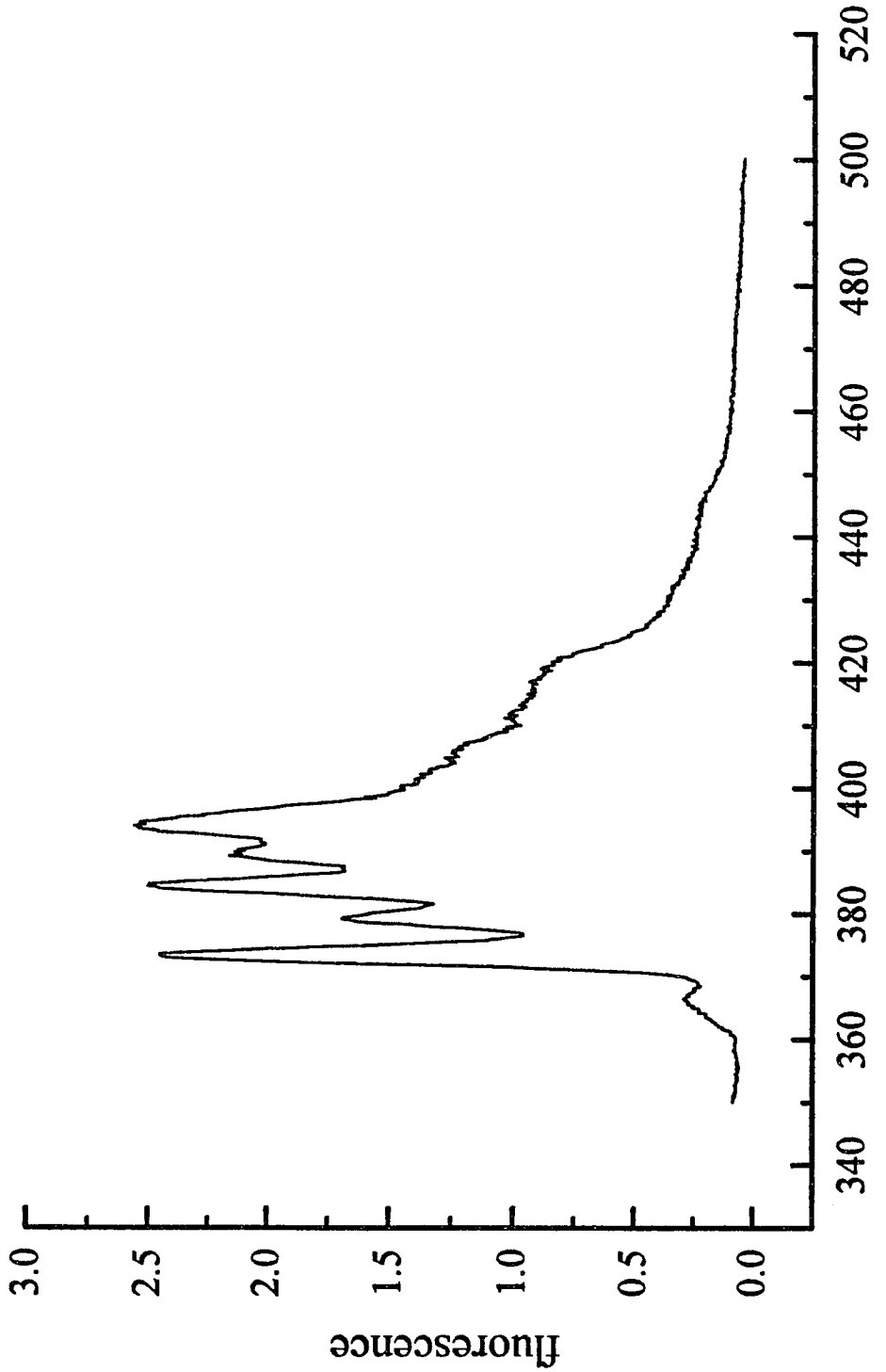


Figure 6-1. Fluorescence Emission Spectrum of 0.05mM Pyrene in 50mM NaDC

Figure 6-2. Fluorescence Emission Spectrum of 0.05mM Pyrene in 50mM Polyoxyethylene-4-dodecyl Ether



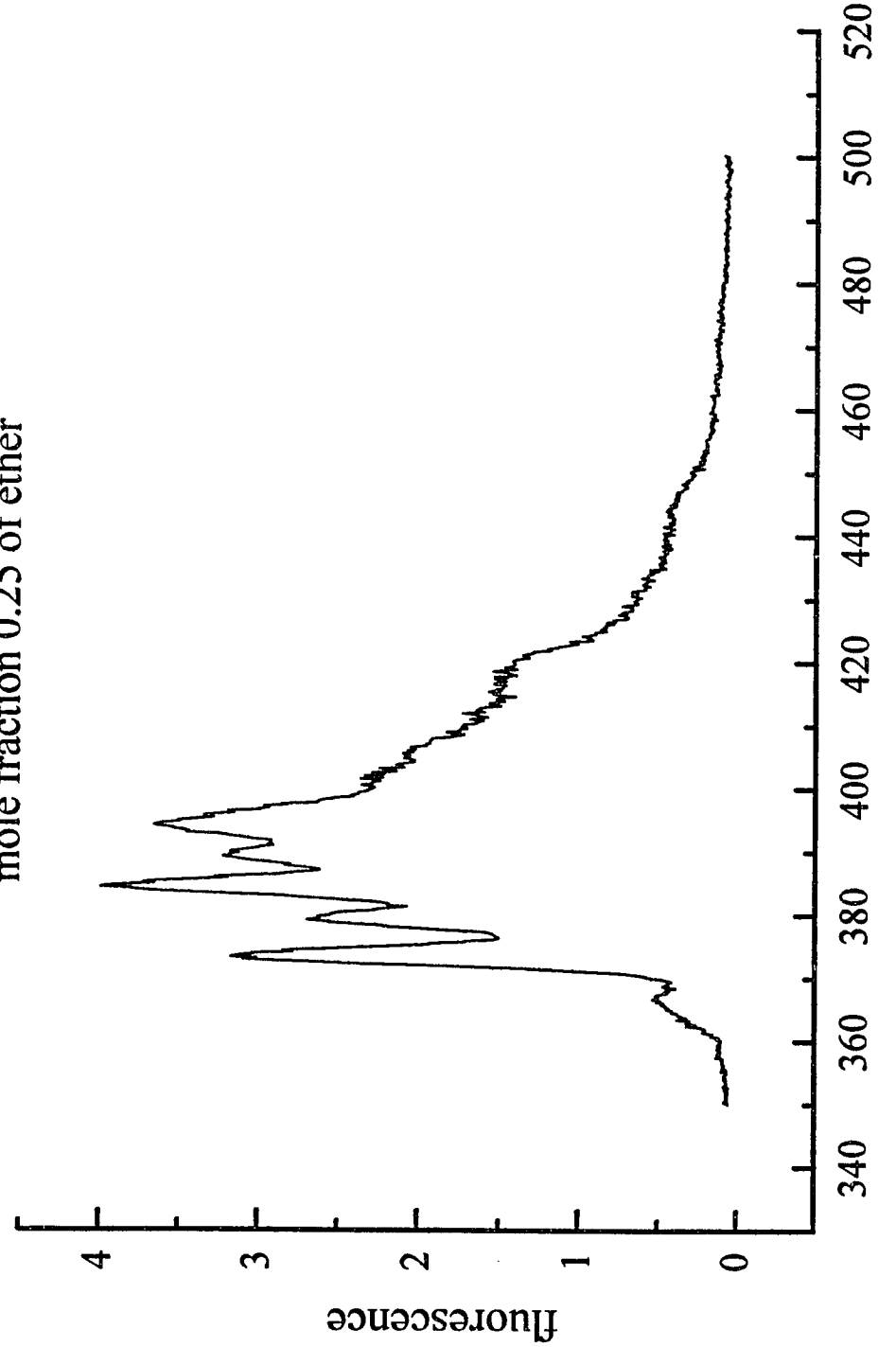


Figure 6-3. Fluorescence Emission Spectrum of 0.05mM Pyrene in a 50mM mixed surfactant solution of NaDC and Polyoxyethylene-4-dodecyl Ether mole fraction 0.25 of ether

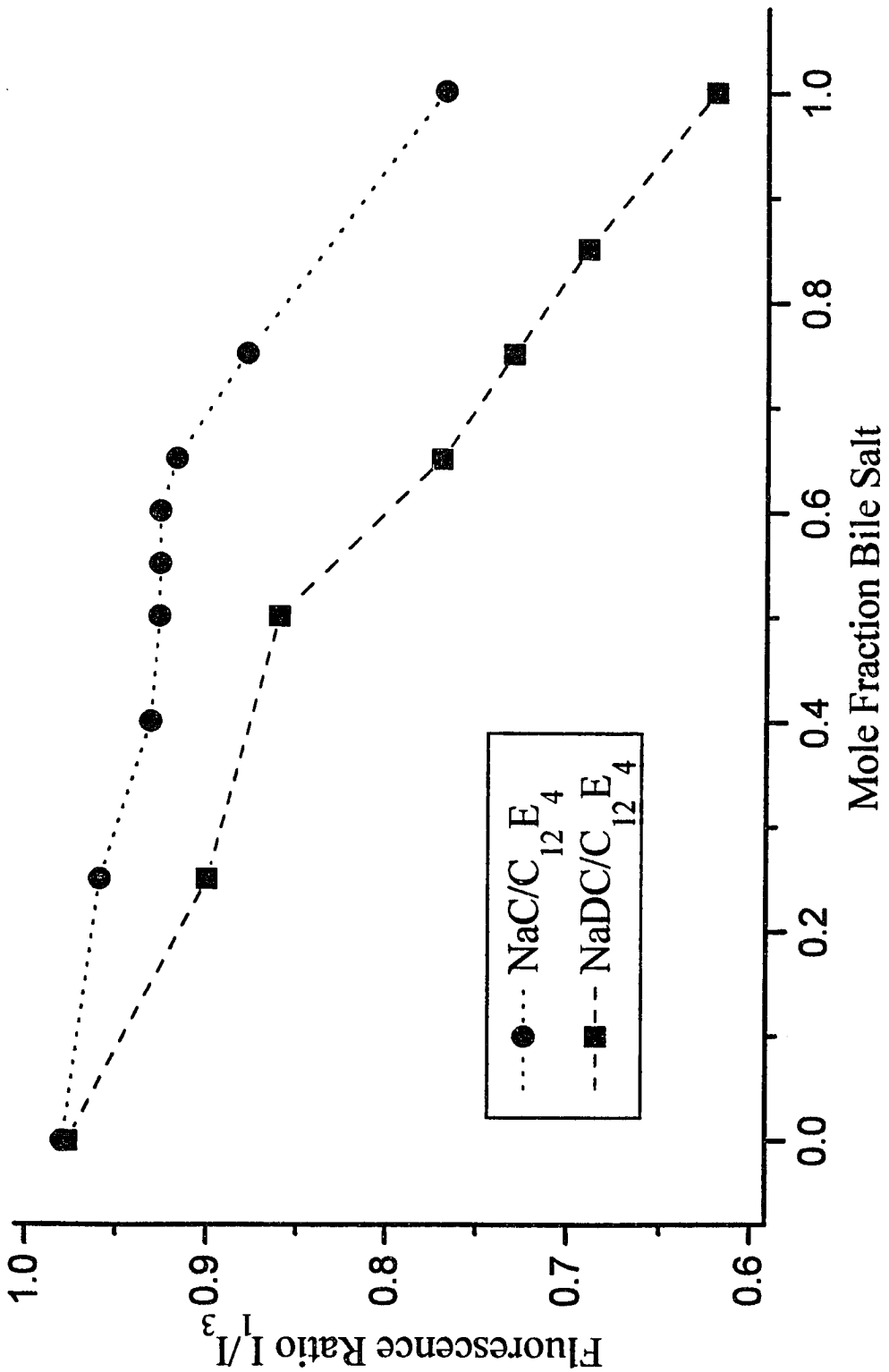


Figure 6-4. Relative Hydrophobicities of Micellar Solutions

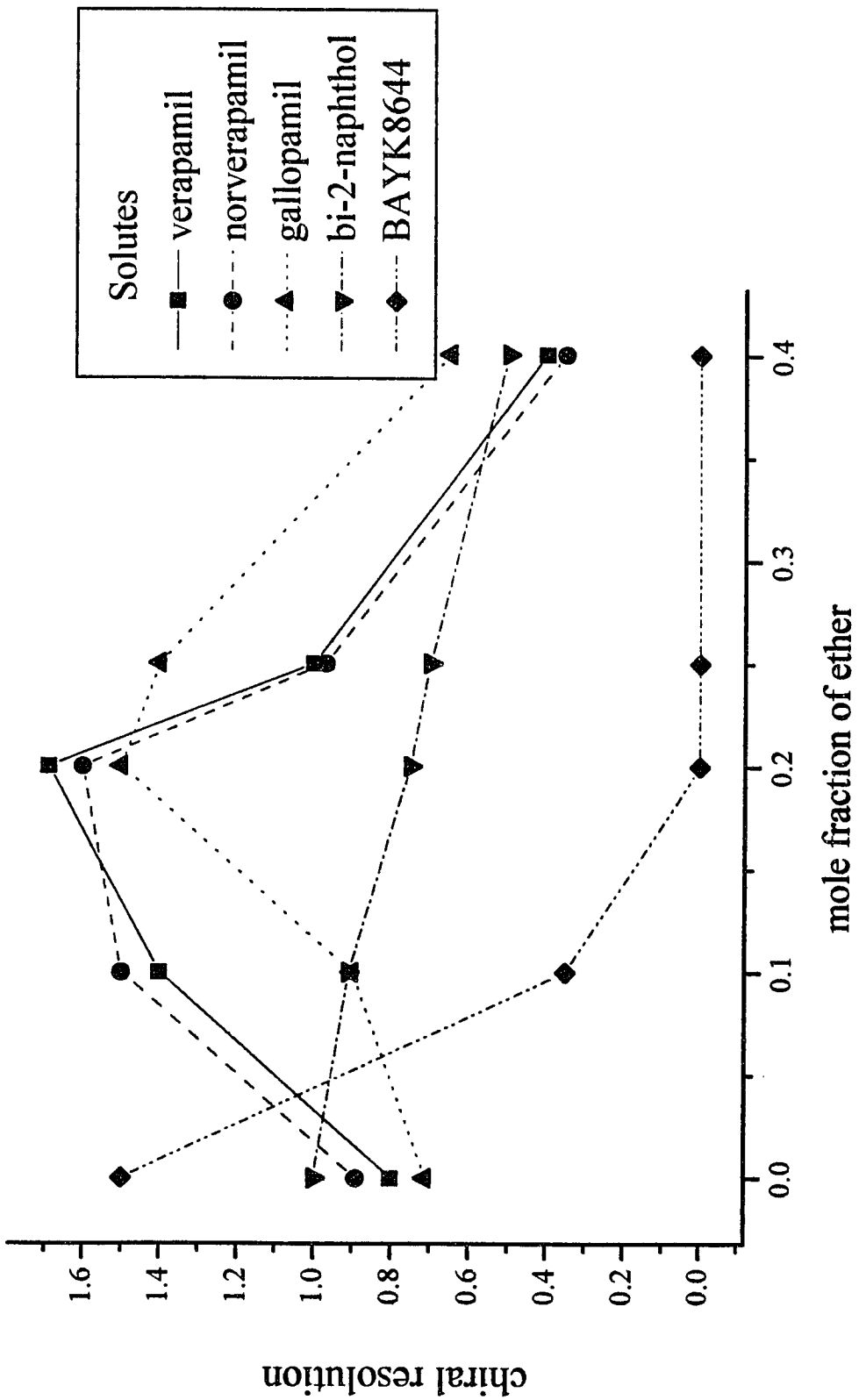


Figure 6-5. Observed Chiral Resolution of Solutes versus Mole Fraction of Ether in MECC: Using solutions of binary mixtures of sodium cholate and polyoxyethylene-4-dodecyl ether.

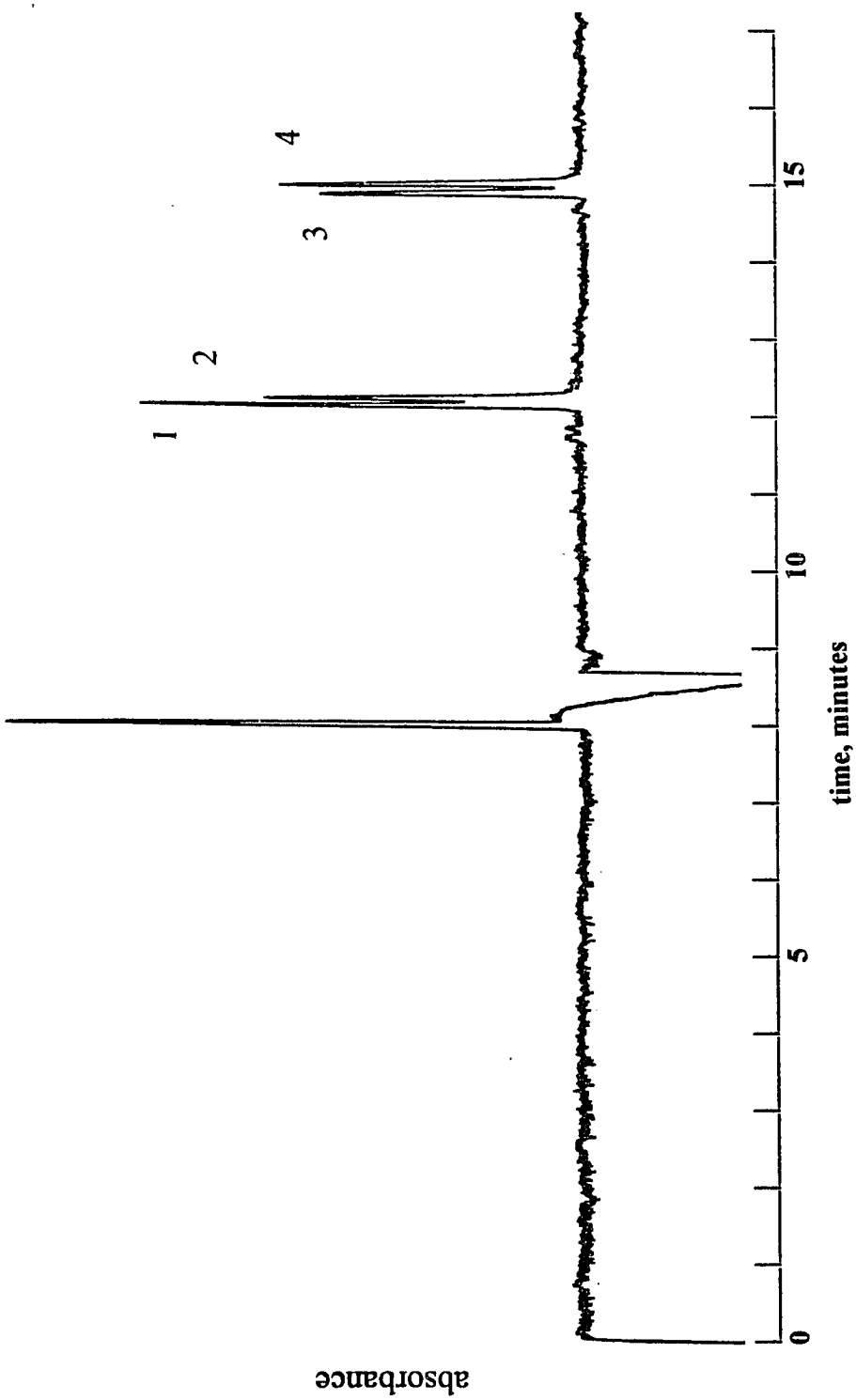


Figure 6-6. Capillary electropherogram of racemic verapamil and bi-2-naphthol using a 50mM NaC solution. Applied voltage 20kV. Peak identifications are as follows: 1) (-)-verapamil, 2) (+)-verapamil, 3) (+)-bi-2-naphthol, and 4) (-)-bi-2-naphthol.

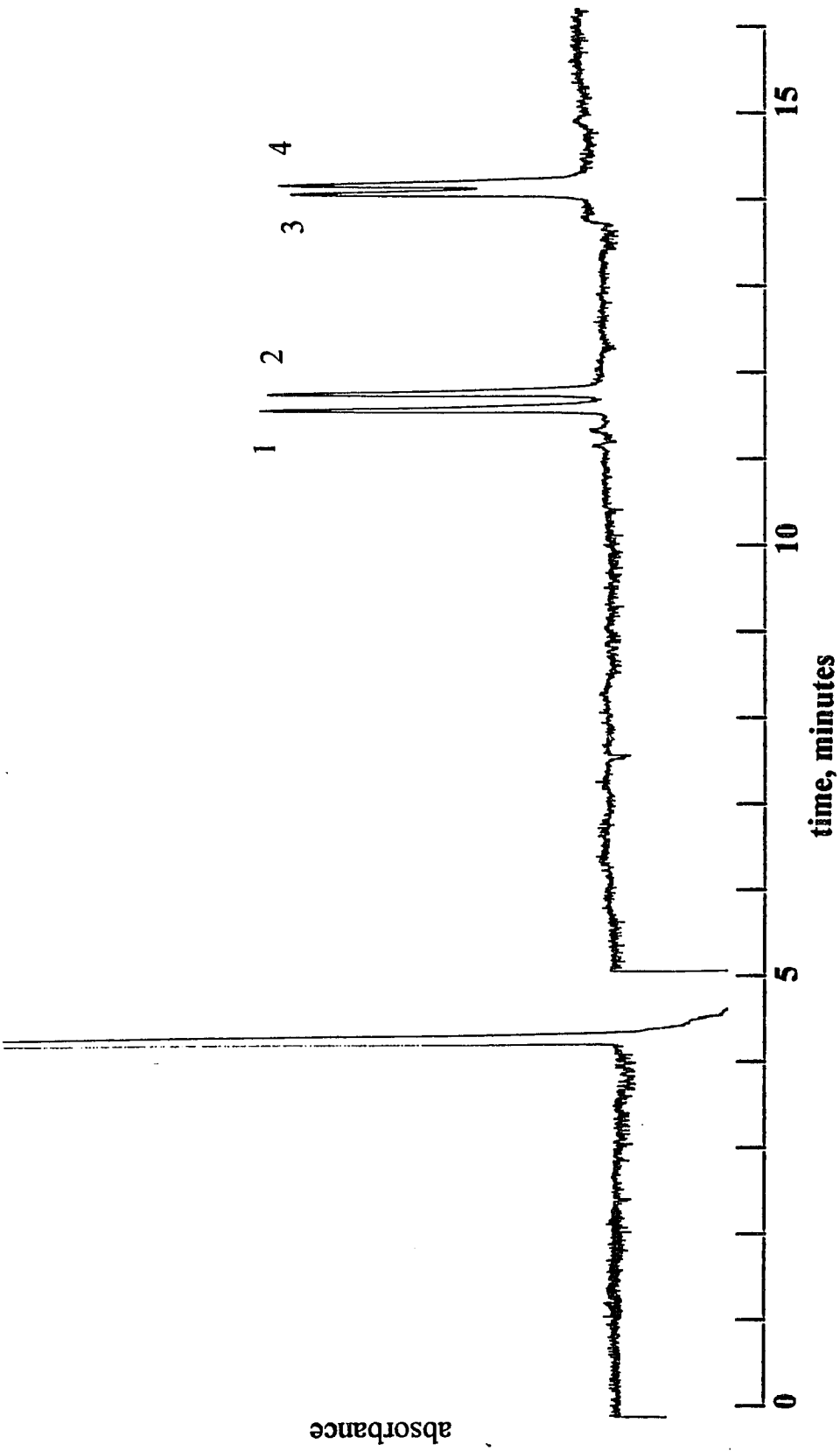
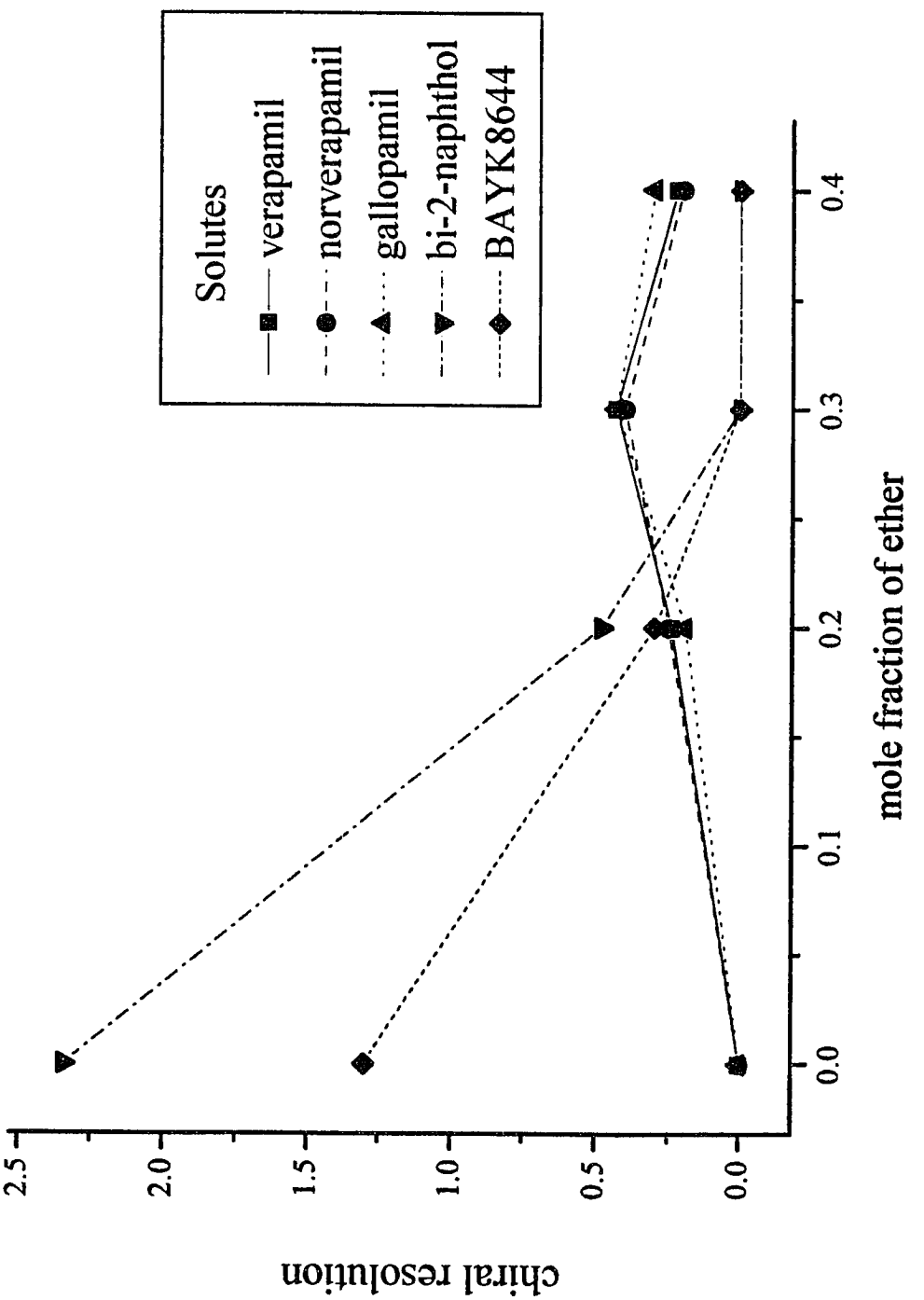


Figure 6-7. Capillary electropherogram of racemic verapamil and bi-2-naphthol using a mixed surfactant solution of NaC and $C_{12}E_4$. The solution contained $C_{12}E_4$ at a mole fraction of 0.20 in a total surfactant concentration of 50mM. Applied voltage 20kV. Peak identifications are as follows: 1) (-)-verapamil, 2) (+)-verapamil, 3) (+)-bi-2-naphthol, and 4) (-)-bi-2-naphthol.

Figure 6-8. Observed Chiral Resolution of Solutes versus Mole Fraction of Ether in MECC: Using solutions of binary mixtures of sodium deoxycholate and polyoxyethylene-4-dodecyl ether.



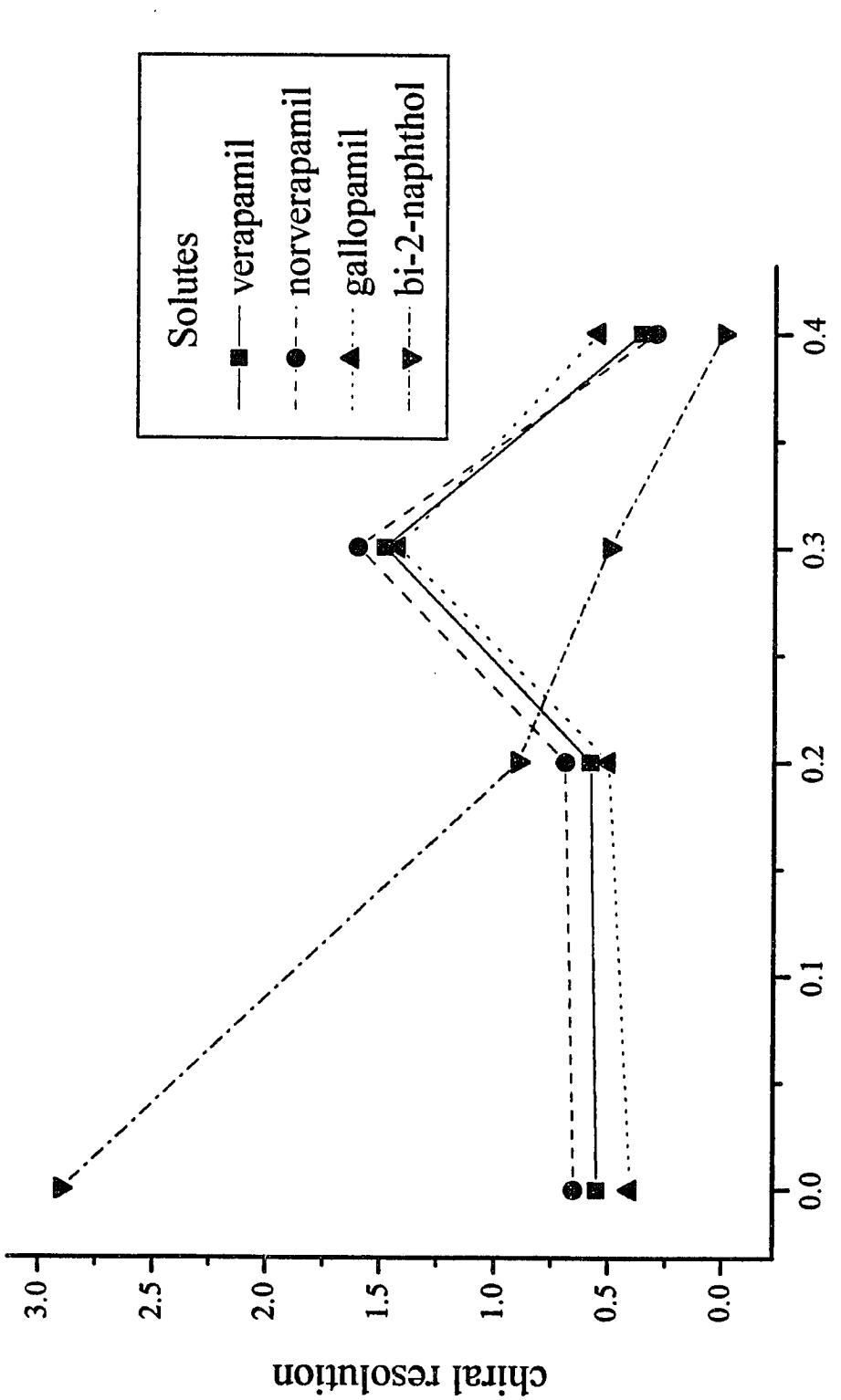


Figure 6-9. Observed Chiral Resolution of Solutes versus Mole Fraction of Ether in MECC: Using solutions of binary mixtures of sodium deoxycholate and polyoxyethylene-4-dodecyl ether containing 25% methanol.

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